IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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| In Re the Application of: | ) | Group Art Unit: 1644 |
| LAHN et al. | ) | Examiner: Schwadron, R.B. |
| Serial No.: 09/826,319 | ) | Confirmation No.: 4155 |
| Filed: April 3, 2001 | ) | |
| Atty. File No.: 2879-80 | ) | **THIRD AMENDED APPEAL BRIEF** |
| For: "METHOD TO INHIBIT AIRWAY HYPERRESPONSIVENESS USING AEROSOLIZED T CELL RECEPTOR ANTIBODIES" | ) | ***SUBMITTED VIA EFS-WEB*** |

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313

Dear Sir:

This Third Amended Appeal Brief is filed in response to Notification of Non-Compliant Appeal Brief mailed August 4, 2008, and in furtherance of the non-final Office Action mailed on April 6, 2007, and the Notice of Appeal filed on July 6, 2007. A Reply to Notification of Non-Compliant Appeal Brief is also submitted herewith. This Third Amended Appeal Brief is being filed with a petition for two months extension. Please charge the appropriate fee to Deposit Account No. 19-1970.

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## I. REAL PARTY IN INTEREST

The real party in interest is **National Jewish Medical and Research Center**, the assignee of record, the Assignment being recorded with the United States Patent Office at Reel/Frame 012070/0760.

## II. RELATED APPEALS AND INTERFERENCES

A prior Appeal Brief was filed in this application on November 20, 2006. The Appeal was dismissed by reopening of prosecution.

## III. STATUS OF CLAIMS

The status of the claims in the application is:

A. TOTAL NUMBER OF CLAIMS IN THE APPLICATION

Claims in the application are: 1-36

B. CURRENT STATUS OF THE CLAIMS:

1. Claims cancelled: 33
2. Claims withdrawn: 3-8
3. Claims pending: 1-32 and 34-36
4. Claims allowed: None
5. Claims rejected: 1, 2, 9-32 and 34-36

C. CLAIMS ON APPEAL

The claims on appeal are: 1, 2, 9-32 and 34-36.

## IV. STATUS OF AMENDMENTS

No amendment was filed subsequent to the final rejection mailed on February 22, 2006 or subsequent to the non-final Office Action mailed April 6, 2007.

## V. SUMMARY OF CLAIMED SUBJECT MATTER

### Claim 1

Claim 1 is directed to method to reduce airway hyperresponsiveness in a mammal that has, or is at risk of developing, airway hyperresponsiveness. (Specification: page 9, lines 2-5; page 18, line 27 to page 19, line 26; page 20, lines 5-17)

The method includes the step of administering to the lungs of the mammal an aerosolized antibody formulation comprising antibodies that selectively bind to a receptor on a T cell selected from: a T cell antigen receptor (TCR) selected from the an αβ TCR and a γδ TCR, CD3, CD4 and CD8. (Specification: page 3, lines 3-19; Table 1; page 11, lines 1-22; page 12, line 4 to page 13, line 11; page 13, lines 12-17; page 37, lines 20-27; Examples 1-6).

The binding of the antibodies to the receptor causes the depletion or inactivation of the T cell. (Specification: page 9, line 23 to page 10, line 9; page 11, lines 24-27; Figs. 2A-2B; Figs. 3A-3B).

The administration of the antibody formulation reduces airway hyperresponsiveness in said mammal. (Specification: page 17, line 18 to page 18, line 26; Example 1, Example 2, Example 3; Figs. 1A-1F)

The administration of the aerosolized antibody formulation affects pulmonary T cell responses in the mammal, while peripheral T cell responses in the mammal are neither substantially stimulated nor substantially inhibited. (Specification: page 10, lines 10-18; Example 5; Fig. 3A-3B).

**Claim 2**

Claim 2 is directed to a method to reduce airway hyperresponsiveness line in a mammal that has, or is at risk of developing, airway hyperresponsiveness. (Specification: page 9, lines 2-5; page 18, line 27 to page 19, line 26; page 20, lines 5-17)

The method includes the step of administering to the lungs of the mammal an aerosolized antibody formulation comprising antibodies that selectively bind to an αβ T cell receptor (TCR). (Specification: page 11, lines 1-22; page 12, lines 4-13 page 13, lines 12-17; page 37, lines 20-27)

The binding of the antibodies to the receptor causes the depletion or inactivation of the T cell. (Specification: page 9, line 23 to page 10, line 9; page 11, lines 24-27; Figs. 2A-2B).

The administration of the antibody formulation reduces airway hyperresponsiveness in said mammal. (Specification: page 17, line 18 to page 18, line 26; Example 1; Figs. 1A-1F)

The administration of the aerosolized antibody formulation affects pulmonary T cell responses in the mammal, while peripheral T cell responses in the mammal are neither substantially stimulated nor substantially inhibited. (Specification: page 10, lines 10-18).

**Claim 16 and Claims 19-23**

Claims 16 and 19-23 are directed to particular embodiments of the invention, wherein said aerosolized antibody formulation is administered at low doses (Specification: Page 10, lines 22-28; page 34, line 9 to page 35, line 6), including a dose of between about 5 μg antibody and about 10 μg antibody per milliliter of formulation (Claim 16), or less than about 40 μg x kilogram$^{-1}$ body weight of the mammal (Claim 19), less than about 1 μg x kilogram$^{-1}$ body weight of the mammal (Claim 20), less than about 0.5 μg x kilogram$^{-1}$ body weight of the

mammal (Claim 21), less than about 0.1 μg x kilogram$^{-1}$ body weight of said mammal (Claim 22), or less than about 20 ng x kilogram$^{-1}$ body weight of the mammal (Claim 23). (Specification: page 6, line 26 to page 7, line 2; page 35, lines 7-28; page 36, lines 20-21; original Claims 16, and 19-23)

**Claim 36**

Claim 36 is directed to method to reduce airway hyperresponsiveness line in a mammal that has, or is at risk of developing, airway hyperresponsiveness. (Specification: page 9, lines 2-5; page 18, line 27 to page 19, line 26; page 20, lines 5-17)

The method includes the step of administering to the lungs of the mammal an aerosolized antibody formulation comprising antibodies that selectively bind to a receptor on a T cell selected from: a T cell antigen receptor (TCR) selected from the an αβ TCR and a γδ TCR, CD3, CD4 and CD8. (Specification: page 11, lines 1-22; page 13, lines 12-17; page 37, lines 20-27)

The binding of the antibodies to the receptor causes the depletion or inactivation of the T cell. (Specification: page 9, line 23 to page 10, line 9; page 11, lines 24-27; Figs. 2A-2B; Figs. 3A-3B).

The administration of the antibody formulation reduces airway hyperresponsiveness in said mammal. (Specification: page 17, line 18 to page 18, line 26; Example 1, Example 2, Example 3; Figs. 1A-1F)

In this method, any stimulation or inhibition of peripheral T cell responses in the mammal after the aerosolized antibody administration is less than about 10% of stimulation or inhibition of the peripheral T cell responses that would be detected if the antibody formulation was administered systemically. (Specification: page 11, lines 14-18).

## VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The sole issues on appeal are:

**A.** Whether Claims 1, 2, 9-32 and 34-46 are unpatentable under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement; and

**B.** Whether Claims 1, 2, 9-32 and 34-46 are unpatentable under 35 U.S.C. § 103(a) over Lobb et al. (U.S. Patent No. 5,871,734) as evidenced by Arrhenius et al. (U.S. Patent No. 5,869,448) in view of Schramm et al. (*Amer. J. Respir. Cell Mol. Biol.*, **22**(2):218-25 (2000)), Wigzell et al. (U.S. Patent No. 5,958,410) and Krause et al. (U.S. Patent Application Publication No. 2002/0037286).

## VII. ARGUMENT

### A. Rejection of Claims 1, 2, 9-32 and 34-46 Under 35 U.S.C. § 112, First Paragraph

Claims 1, 2, 9-32 and 34-46 have been once rejected under 35 U.S.C. § 112, first paragraph, on the basis that the claims allegedly fail to meet the requirements of written description.

The rejection reasons that the claims encompass the use of antibodies that bind to T cell receptors (TCR), CD3, CD4 and CD8 from thousands of mammalian species. The rejection argues that while human and mouse counterparts of these molecules were known, there are thousands of counterparts from other mammalian species that were not known and have not been sequenced at the amino acid level. The rejection contends that the skilled artisan cannot envision the detailed structure of the encompassed antibodies and therefore, that conception has not been achieved until reduction to practice has occurred. Further, it is stated that in the instant application, the peptide itself is required. The rejection reasons, "if an inventor is 'unable to envision the detailed constitution of a gene so as to distinguish it from other materials…conception has not been achieved until reduction to practice has occurred'". As controlling precedent, the rejection references: *Regents of the University of California v. Eli Lilly*, 119 F.3d 1559, 1568, 43 USPQ2d 1398, 1406 (Fed. Cir. 1997); *Amgen, Inc. v. Chugai Pharmaceutical*, 927 F.2d 1200, 1206, 18 USPQ2d 1016, 1021 (Fed. Cir. 1991); and *Fiers v. Revel*, 984 F.2d 1164, 1168, 25 USPQ2d 1601, 1604-05 (Fed. Cir. 1993). See the Office Action mailed April 6, 2007, pages 2-3.

The first paragraph of Section 112 of Title 35 of the United States Code requires that:

> "The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention."

To satisfy the written description requirement of this paragraph, a patent specification must describe the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention. See, e.g., *Moba, B.V. v. Diamond Automation, Inc.*, 325 F.3d 1306, 1319, 66 USPQ2d 1429, 1438 (Fed. Cir. 2003); *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d at 1563, 19 USPQ2d at 1116 ("applicant must...convey to those skilled in the art that, as of the filing date sought, he or she was in possession of the invention"). What is conventional or well known to one of ordinary skill in the art need not be disclosed in detail. See *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d at 1384, 231 USPQ at 94. See also *Capon v. Eshhar*, 418 F.3d 1349, 1357, 76 USPQ2d 1078, 1085 (Fed. Cir. 2005). From *Falkner v. Inglis*, 448 F.3d 1357, 1366, 79 USPQ2d 1001, 1007 (Fed. Cir. 2006): "(1) examples are not necessary to support the adequacy of a written description; (2) the written description standard may be met...even where actual reduction to practice of an invention is absent; and (3) there is no per se rule that an adequate written description of an invention that involves a biological macromolecule must contain a recitation of known structure".

Based on the current legal precedent regarding written description, Appellant contends that Claims 1, 2, 9-32 and 34-46 meet the written description requirement of 35 U.S.C. § 112, first paragraph. Arguments are presented below for each claim or group of claims. Claims argued under different subheadings below do not stand or fall together.

**Claims 1, 9-32, and 34-46**

Appellants submit that the rejection of the claims on the basis of failure to meet the written description requirement is improper and contravenes the current legal precedent. Specifically, the rejection reasons that the skilled artisan cannot envision the detailed structure of the antibodies encompassed by the claims because specifically, a peptide is allegedly required (apparently a peptide of an αβ T cell receptor (TCR), a γδ TCR, CD3, CD4 or CD8). Therefore, the rejection states that conception has not been achieved until reduction to practice has occurred. Appellants submit that the rejection is requiring that the specification teach what is conventional or well-known in the art with respect to the recited proteins and antibodies, which is contrary to court decisions more recent than those cited in the rejection. The more recent legal precedent clarifies the standard for written description.

More particularly, the rejection refers to case law in which the discovery of a gene function or structure itself was at issue. For example, in *Regents of the University of California v. Eli Lilly*, 119 F.3d 1559, 1568, 43 USPQ2d 1398, 1406 (Fed. Cir. 1997), the claims at issue recited a cDNA encoding human insulin, where only a cDNA encoding rat insulin had been described. *Amgen, Inc. v. Chugai Pharmaceutical*, 927 F.2d 1200, 1206, 18 USPQ2d 1016, 1021 (Fed. Cir. 1991) concerns a claim to a gene encoding human erythropoietin, where such gene had not yet been cloned or sequenced. *Fiers v. Revel*, 984 F.2d 1164, 1168, 25 USPQ2d 1601, 1604-05 (Fed. Cir. 1993) concerns claims to a gene encoding human fibroblast interferon-beta polypeptide, who was the first to invent the isolated sequence, and specifically, that conception of the sequence did not occur until the sequence was obtained.

However, the fact patterns in the cases cited by the rejection do not apply to the fact pattern in the present application, which claims a new use for a molecule that was already known in the art. Specifically, Appellants are not claiming the discovery of any of an αβ T cell receptor (TCR), a γδ TCR, CD3, CD4 and CD8, nor antibodies that bind to such proteins, but rather, a novel method of using such antibodies. It is submitted that the present specification provides a written description of this invention that is sufficient to establish that the inventors were in possession of the invention at the time of filing.

> "The 'written description' requirement must be applied in the context of the particular invention and the state of the knowledge...When the prior art includes the nucleotide information, precedent does not set a *per se* rule that the information must be determined afresh." *Capon v. Eshhar*, 418 F.3d 1349, 1357, 76 USPQ2d 1078, 1085 (Fed. Cir. 2005).

Indeed, as stated in *Capon v. Eshhar, ibid.*:

> "None of the cases to which the Board attributes the requirement of total DNA re-analysis, i.e., Regents v. Lilly, Fiers v. Revel, Amgen, or Enzo Biochem, require a re-description of what was already known. In *Lilly, 119 F.3d at 1567*, the cDNA for human insulin had never been characterized. Similarly in *Fiers, 984 F.2d at 1171*, much of the DNA sought to be claimed was of unknown structure, whereby this court viewed the breadth of the claims as embracing a "wish" or research "plan." In *Amgen, 927 F.2d at 1206*, the court explained that a novel gene was not adequately characterized by its biological function alone because such a description would represent a mere "wish to know the identity" of the novel material. In *Enzo Biochem, 296 F.3d at 1326*, this court reaffirmed that deposit of a physical sample may replace words when description is beyond present scientific capability. In *Amgen Inc. v. Hoechst Marion Roussel, Inc., 314 F.3d 1313, 1332 (Fed. Cir. 2003)* the court explained further that the written description requirement may be satisfied "if in the knowledge of the art the disclosed function is sufficiently correlated to a particular, known structure.""

Appellants submit that the same reasoning used in *Capon v. Eshhar* should be applied to the present claims. Indeed, all of the *same* proteins that are subject of the present rejection were included in the written description rejection at issue in *Capon v. Eshhar* (*i.e.,* the α, β, γ or δ chain of a T cell receptor, CD3, CD4 and CD8).

The present specification teaches that each of the proteins recited in the claims were known in the art at the time of the invention, and further teaches that antibodies binding to each of the cited proteins were known in the art at the time of the invention, including antibodies to the murine and human forms of the proteins, with provision of at least one commercial source for such antibodies. More particularly, the specification teaches on page 3, lines 3-19, that antibodies against the T cell antigen receptor (TCR), CD3, and CD4 were known and references publications and patents that describe antibodies against CD3, the TCR α or β chains, and CD4. Table 1 provides a list of antibodies that were in clinical application at the time of the invention, including antibodies against CD3 and CD4, and including humanized antibodies. Page 13, lines 12-17 teaches that "Antibodies against various T cell receptors useful in the present invention are known in the art. For example, antibodies against murine TCR-β, TCR-δ, and TCR-Vγ1 are described in the examples section. Antibodies against murine and human TCR-β, TCR-α, TCR-δ, TCR-γ, CD3, CD8 and CD4 are known in the art and are publicly available and referenced through Pharmingen (San Diego, CA), for example." Examples 1-6 provide examples of antibodies against TCR-αβ and TCR- γδ. The state of the art at the time of the invention was such that each of the recited proteins and antibodies directed against such proteins were known. Indeed, the rejection acknowledges that the murine and human counterparts of these molecules were known at the time of the invention.

However, the rejection implies that in order to meet the written description requirement, the specification must describe each and every permutation of an antibody that binds to such proteins, including all proteins from every mammalian species. Appellants disagree. The specification describes the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventors had possession of the claimed invention.

Referring again to *Capon v Eshhar*:

> "It is not necessary that every permutation within a generally operable invention be effective in order for an inventor to obtain a generic claim, provided that the effect is sufficiently demonstrated to characterize a generic invention. See *In re Angstadt, 537 F.2d 498, 504 (CCPA 1976).*"

The specification provides not only general teachings regarding the antibodies and proteins to which they bind, but also working examples describing the invention using several different antibodies, and references specific examples of such antibodies in the scientific and patent literature, as well as commercially available antibodies covering all of the claimed embodiments.

> "Eli Lilly does not set forth a per se rule that whenever a claim limitation is directed to a macromolecular sequence, the specification must always recite the gene or sequence, regardless of whether it is known in the prior art", and "Indeed, the forced recitation of known sequences in patent disclosures would only add unnecessary bulk to the specification. Accordingly we hold that where, as in this case, accessible literature sources clearly provided, as of the relevant date, genes and their nucleotide sequences…satisfaction of the written description requirement does not require either the recitation or incorporation by reference…of such genes and sequences. *Falkner v. Inglis*, 448 F.3d 1357, 1366, 79 USPQ2d 1001, 1007 (Fed. Cir. 2006).

Current case law also further addresses written description with respect to antibodies. "Disclosure of an antigen fully characterized by its structure, formula, chemical name, physical properties, or deposit in a public depository provides an adequate written description of an antibody claimed by its binding affinity to that antigen." *Noelle v. Lederman*, 355 F.3d 1343, 1349, 69 USPQ2d 1508, 1514 (Fed. Cir. 2004). In *Noelle v. Lederman*, a generic claim to an antibody was deemed not to meet the written description requirement because only the mouse version of the protein to which it bound was known. In contrast, in the present case, and at a minimum by the rejections' own reasoning, both human and mouse antigens were known at the time of the invention. A brief review of a few of the patents described on page 3 of the present specification, *e.g.,* U.S. Patent No. 6,171,799 (describing in detail both αβ TCR and γδ TcR), shows that the art at the time of the invention had detailed knowledge of the structure of these proteins, as well as antibodies that bind to these proteins. Thus, at the time of the invention, the antigens to which the recited antibodies bind had been fully characterized and were known in the art with respect to many different mammalian species.

Accordingly, it is submitted that the present specification meets the written description requirement, and Appellants respectfully request that the Board withdraw the rejection of Claims 1, 9-32, and 34-46 under 35 U.S.C. § 112, first paragraph.

**Claim 2**

With respect to Claim 2, Appellant notes that this claim is directed to the elected species of αβTCR, whereas Claim 1 in the group of claims above is directed to all species, including non-elected species that were previously rejoined in the Office Action mailed September 8, 2005, and then subsequently restricted again in the Office Action mailed February 22, 2006.

Appellant's arguments against the rejection of Claim 2 under 35 U.S.C. § 112, first paragraph, are essentially the same as the arguments presented above in view of Claim 1, although such arguments are in the case of Claim 2 directed exclusively to the elected species. However, in the event that Claim 1 falls as a result of consideration of non-elected species in Claim 1, Appellant expressly submits that Claim 2, as well as dependent Claims 9-15, 17-18, and 24-35, to the extent they depend from Claim 1 with respect to the elected invention of αβTCR as recited in Claim 2, do not stand or fall together with Claim 1.

With particular regard to αβTCR, it is Appellants' position that αβTCR proteins were well known in the art at the time of the invention, as discussed in detail in the paragraphs above, and accordingly, were fully characterized to the point that an antibody that binds to an αβTCR was also well known. Moreover, "It is not necessary that every permutation within a generally operable invention be effective in order for an inventor to obtain a generic claim, provided that the effect is sufficiently demonstrated to characterize a generic invention. See *In re Angstadt, 537 F.2d 498, 504 (CCPA 1976).*" The working examples of the specification describe the use of an antibody that binds to αβTCR in the method of the invention, and it is believed that the specification has fully met the written description requirement of 35 U.S.C. § 112, first paragraph.

In view of the foregoing discussion, Appellants respectfully request that the Board withdraw the rejection of Claim 2 under 35 U.S.C. § 112, first paragraph.

**B. Rejection of Claims 1, 2, 9-32 and 34-46 Under 35 U.S.C. § 103(a)**

Claims 1, 2, 9-32 and 34-46 have been at least twice rejected under 35 U.S.C. § 103(a) over Lobb et al. (U.S. Patent No. 5,871,734) as evidenced by Arrhenius et al. (U.S. Patent No.

5,869,448) in view of Schramm et al. (*Amer. J. Respir. Cell Mol. Biol.*, 22(2):218-25 (2000)), Wigzell et al. (U.S. Patent No. 5,958,410) and Krause et al. (U.S. Patent Application Publication No. 2002/0037286).

The rejection reasons that it would have been *prima facie* obvious to one of ordinary skill in the art at the time of the invention to create the claimed invention because Lobb et al. teach aerosol administration of an antibody that binds T cells to treat asthma and Schramm et al. teach that intravenous (i.v.) administration of a different antibody that binds T cells (anti-TCR αβ) can be used to treat asthma. Motivation is alleged because Lobb et al. teach that the antibody can be administered in a variety of art known routes including aerosol. Motivation is further alleged on the basis that Krause et al. teach that antibodies that inhibit T cell activation are preferably administered by pulmonary aerosol and Wigzell et al. teach that pathologic T cells found in the lungs can be treated via intrapulmonary administration of anti-TCR antibody. It is further reasoned that a neutralizing antibody would be used in the claimed method because Schramm et al. teach that asthma symptoms are reduced in the absence of TCRαβ T cells. It is reasoned that with respect to the particular recited dosages of formulation or dosage per weight, a "routineer" would initially test a wide variety of different dosages in order to determine the smallest effective dosage, that the antibody would be administered by a "routineer in conjunction with art known treatments for asthma, and that the antibody would have been administered either before or during asthma symptoms. Finally, the rejection reasons that Lobb et al. teach that the effect can be achieved without detectable blood levels of antibody wherein the aerosol administered antibody would therefore not substantially effect peripheral immune T cell responses. See, *e.g.*, Office Action mailed April 6, 2007 (April 6 Office Action), page 5, which is repeated on page 6.

With respect to the individual references, the rejection further reasons that Lobb et al. teach the use of antibody against VLA-4 to treat asthma, and Arrhenius et al. is cited as teaching that VLA-4 is a receptor on T cells. Lobb et al. is further cited for the following teachings: airway hyperresponsiveness occurs in asthma; the use of humanized anti-VLA-4 antibody and a monovalent antibody; the anti-VLA4 antibody does not stimulate T cell activation (via an alleged teaching that the antibodies inhibit VLA-4 function); the use of antibody dosages encompassed in the instant Claims 18 and 19; administration of antibody by a nebulized spray; the method of instant Claim 27; the methods of instant Claims 28, 31 and 32; "Lobb et al. teach that the effect seen can be achieved without detectable blood levels of antibody (see column 12, last paragraph) wherein the antibody would not therefore substantially effect peripheral immune function (e.g. because it was not present in the blood)"; the use of the method in humans; and that the method resulted in a 70% decrease in inhibition of late phrase response. The rejection also states that "Lobb et al. disclose: 'For instance, to the extent that the beneficial effects reported herein are due to the inhibition of leukocyte recruitment to VCAM-1 expressing endothelium...' (column 8, last paragraph):, in support of the argument that Lobb et al. contemplate that their method involves inhibition of leukocytes including T cells. The rejection acknowledges that Lobb et al. does not teach the use of anti-TCRαβ antibodies. Schramm et al. is cited for an alleged teaching of the use of intravenous anti-TCR antibodies to treat asthma. The rejection argues that there is no teaching in Schramm et al. that a complete systemic depletion of an entire T cell subset form an animal is required in the antibody treated animals. Krause et al. is cited as teaching that antibodies that inhibit T cell activation are preferably

administered by pulmonary aerosol. Wigzell et al. is cited as teaching that pathologic T cells found in the lungs can be treated via intrapulmonary administration of anti-TCR antibody.

To establish a *prima facie* case of obviousness: (1) First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings; (2) Second, there must be a reasonable expectation of success; (3) Third, the prior art reference (or references when combined) must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on applicant's disclosure. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991). See also MPEP § 2143-§2143.03. An Examiner has the initial burden of establishing a *prima facie* case of obviousness before the burden shifts to the applicant to show otherwise. See, e.g., In re Fine, 5 U.S.P.Q.2d 1596, 1598 (Fed. Cir. 1988). In determining obviousness, one must focus on Applicant's invention as a whole. *Symbol Technologies Inc. v. Opticon Inc.*, 19 U.S.P.Q.2d 1241, 1246 (Fed. Cir. 1991). The primary inquiry is:

> "whether the prior art would have suggested to one of ordinary skill in the art that this process should be carried out and would have had a reasonable likelihood of success . . . . Both the suggestion and the expectation of success must be found in the prior art, not in the applicant's disclosure." *In re Dow Chemical*, 5 U.S.P.Q.2d 1529, 1531 (Fed. Cir. 1988).

Based on the required showing set forth by the Federal Circuit, Appellant contends that a *prima facie* case of obviousness has not been established in the present case. Arguments are

presented below for each claim or group of claims. Claims argued under different subheadings below do not stand or fall together.

**Claims 1, 9-15, 17-18, and 24-35**

Appellant submits that the combination of references fails to teach or suggest the use of an *aerosolized antibody* having one of the particularly recited receptor specificities (*i.e.,* αβ TCR, γδ TCR, CD3, CD4 or CD8) to *reduce airway hyperresponsiveness*, wherein the binding of the antibody to the receptor *causes the depletion or inactivation of the T cell* and wherein the administration of the aerosolized antibody formulation *affects pulmonary T cell responses in the mammal, while peripheral T cell responses in the mammal are neither substantially stimulated nor substantially inhibited.* It is also Appellant's position that the combination of references fails to provide the requisite motivation to combine the references to arrive at the claimed invention and further, fails to provide a reasonable expectation of success to arrive at the claimed invention as claimed in Claims 1, 9-15, 17-18, and 24-35.

The rejection is primarily based on the combination of Lobb et al. and Schramm et al., where the rejection asserts that Lobb et al. teach aerosol administration of an antibody that binds T cells to treat asthma and Schramm et al. teach that a different antibody that binds T cells (anti-TCRαβ) can be used to treat asthma. However, Appellant submits that this combination, even when combined with the other references of Krause et al., Arrhenius et al. and Wigzell et al., fail to teach or suggest the invention as claimed in Claims 1, 9-15, 17-18, and 24-35. Furthermore, there is no suggestion or motivation found in the references themselves or in the art at the time of the invention to make the combination as the rejection has done. It is the rejection's apparent position that it would be obvious to substitute the antibody of Schramm et al. into a method of

Lobb et al., and that based on the teachings of Wigzell et al., Krause et al. and Schramm et al., one of skill in the art would be motivated to do so and would expect success in making the substitution. However, Appellant submits that there is no teaching, suggestion or motivation provided by any of the cited references to substitute the anti-VLA-4 antibody of Lobb et al. with the anti-TCRαβ antibody of Schramm et al., or *vice versa*, even when combined with the other three references. Indeed, neither of Lobb et al. or Schramm et al. attempts to extend its teachings beyond the specific antibody having the specific specificity described in the respective reference.

Obviousness cannot be established by combining the teachings of the prior art to produce the claimed invention, absent some teaching or suggesting supporting the combination. ACS Hospital Systems v. Montofiore Hospital, 221 USPQ 929, 933 (Fed.Cir. 1974). "A statement that modifications of the prior art to meet the claim limitations would have been 'well within the ordinary skill of the art at the time the invention was made', because the cited references relied upon teach that all aspects of the claimed invention were individually known in the art is not sufficient to establish a *prima facie* case of obviousness without some objective reason to combine the teachings of the references. *Ex parte Levengood*, 28 USPQ2d 1300 (Bd. Pat. App. & Inter. 1993)." MPEP 2143.01.

With these standards in mind, first, Appellant reviews the rejection's allegation that Lobb et al. teach aerosol administration of an antibody that binds T cells to treat asthma, and the use of this alleged teaching as a basis for including Lobb et al. in the combination of cited references. Lobb et al. is directed to the use of antibodies recognizing VLA-4 integrin, which is a cell adhesion receptor that binds to adhesion molecules such as VCAM-1 (see Arrhenius et al., col. 1, line 54 to col. 2, line 11). Appellant agrees with the rejection that Arrhenius et al. teaches that

VLA-4 is a receptor found on T cells, which is all that Arrhenius is alleged to contribute to the combination of references. However, Appellant further notes, as supported by Arrhenius et al. (col. 1, lines 54-65), and by Lobb et al. (col. 2, lines 60-62), anti-VLA4 binds to *a variety of cell types* in addition to T cells, including B lymphocytes, natural killer cells, monoctyes, basophils and eosinophils. This teaching is relevant to the interpretation of the teachings of Lobb et al., as discussed below. Claim 1 of the present invention is directed to the use of aerosolized antibodies that selectively bind to a receptor on a T cell selected from: an αβ T cell antigen receptor (TCR), a γδ TCR, CD3, CD4 and CD8 (which are all *T cell-specific* receptors), to reduce airway hyperresponsiveness in a mammal.

One important aspect of the rejection reasons that because Lobb et al. teach that anti-VLA4 "treats asthma" in a mammal, and because VLA4 is found on T cells, Lobb et al. accordingly provide a teaching sufficient to combine this reference with a second reference (Schramm et al.) that describes a *different* antibody that binds to a *different* receptor on T cells, expecting that such different antibody will also be useful in the method of Lobb et al. First, it is Appellant's position that one of skill in the art reviewing Lobb et al. would not conclude that the effects on airway hyperresponsiveness observed in Lobb et al. are due to an action of the antibody on T cells, since that conclusion is not presented in Lobb et al. "In determining the propriety of the Patent Office case for obviousness in the first instance, it is necessary to ascertain whether or not the reference teachings would appear to be sufficient for one of ordinary skill in the relevant art having the reference before him to make the proposed substitution, combination, or other modification." *In re Linter*, 458 F.2d 1013, 1016, 173 USPQ 560, 562 (CCPA 1972).

Appellant submits that there is absolutely no teaching or even a suggestion in Lobb et al. that the effects of the anti-VLA4 on airway hyperresponsiveness or any other aspect of asthma, *regardless* of whether the antibody can bind to T cells, are due to any action of the antibody on T cells (*i.e.,* T lymphocytes). Initially, it is noted that a variety of cell types, including basophils, eosinophils, lymphocytes, neutrophils, and monocytes, including macrophages, can be generically referred to as leukocytes or white blood cells, leukocytes being the general term used in Lobb et al. Lymphocytes are a specific subset of leukocytes and include T and B lymphocytes. It is Appellant's position that Lobb et al. teach that the observed effects of anti-VLA4 was on neutrophil and eosinophil recruitment. Indeed, the data of Lobb et al. do not indicate that anti-VLA4 had *any* effect on lymphocyte numbers or recruitment, and upon review of Fig. 4 of Lobb et al., it appears as though anti-VLA4 actually *increased* the lymphocytes in the lungs of the animal (Fig. 4B of Lobb et al.). Lobb et al. provide no other relevant discussion of lymphocytes and no specific mention of T lymphocytes in the patent. However, Lobb et al. provide a *clear teaching* that anti-VLA4 administration caused a significant inhibition of the recruitment of neutrophils and eosinophils to the lung (column 3, lines 4-7; col. 8, line 64 to col. 9, line 2 (note in particular the reference to blocking interactions with *endothelial* cell receptor molecules); Figure 4, and column 12, lines 10-21), which are the only leukocytes to which Lobb et al. appear to attribute the inhibition of the various observed responses. Lobb et al. specifically teach that binding of anti-VLA-4 to leukocytes (*e.g.*, eosinophils and neutrophils) inhibits the migration of such cells to VCAM-1 expressing endothelium, and propose that "antibodies that interfere with the adhesion between leukocyte antigens and *endothelial cell* receptor molecules may be advantageous" (emphasis added) (col. 8, line 63 to col. 9, line 2). Again, as discussed

above, Lobb et al., as supported by Arrhenius et al., teach that VLA-4 is present on a variety of different cell types, including eosinophils, which are important mediators of inflammation in asthma. Accordingly, at a minimum, the effects of anti-VLA-4 appear from Lobb et al. to be largely attributable to the action of the antibody on neutrophils and eosinophils, and there is no teaching or suggestion in Lobb et al., explicit or implicit, that the anti-VLA-4 antibody depleted or inactivated T lymphocytes, or that an effect on T cells contributed at all to the observed responses after administration of the antibody.

In contrast, the antibody of the present invention, which binds to a T cell-specific receptor, removes (depletes) and/or inactivates a small and relevant population of T cells which are directly involved in the allergic inflammatory response in the lung. Lobb et al. do not teach or suggest any antibody other than one that binds to VLA-4 or LFA3, which are both adhesion molecules, nor any other mechanism of inhibiting allergic inflammation other than inhibiting the migration of leukocytes (specifically, neutrophils and eosinophils) to lung tissue. Therefore, Lobb et al. can not provide motivation to switch to a different antibody or different mechanism of action, including the one taught by Schramm et al., even when viewed with Wigzell et al. and Krause et al.

Accordingly, it is Appellant's position that Lobb et al. do not teach an aerosolized antibody that binds to a T cell receptor and causes the depletion or inactivation of the T cell, nor do Lobb et al. teach that one should modulate T cells to treat asthma, nor would the teachings of Lobb et al. motivate one of skill in the art to look at the modulation of T cells to treat asthma or airway hyperresponsiveness. At best, the teachings of Lobb et al. would suggest that one should look at methods of targeting *eosinophils or neutrophils* to treat asthma, and could further suggest

that modulation of T cells is not necessary, or is not effective using an anti-VLA4 antibody. This is a *teaching away* from the present invention. Indeed, the only connection between the anti-VLA4 and an action on T cells appears to come from the rejection, and not the teachings of Lobb et al., and would therefore appear to be based on the teachings of the instant specification. The rejection therefore appears to use hindsight in making the obviousness rejection in that the rejection attempts to find each element of the pending claims in the prior art, and then reasons that it is easy to reassemble these elements into the invention; however "it is impermissible to use the claimed invention as an instruction manual or "template" to piece together the teaching of the prior art so that the claimed invention is rendered obvious." *In re Fritch*, 972 F.2d 1260, 1266, 23 USPQ2d 1780, 1784 (Fed. Cir. 1992). The invention must be viewed not with the blueprint drawn by the inventor, but in the state of the art that existed at the time." *Interconnect Planning Corp. v. Feil*, 774 F2d 1132,1138, 227 USPQ 543,547 (Fed. Cir. 1985). "As is clear from cases such as *Adams*, a patent composed of several elements is not proved obvious merely by demonstrating that each of its elements was, independently, known in the prior art. Although common sense directs one to look with care at a patent application that claims as innovation the combination of two known devices according to their established functions, it can be important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does." *KSR International Co. v. Teleflex Inc. (KSR), 127 S. Ct. 1727*; *167 L. Ed. 2d 705*; 82 USPQ2d 1385 (2007).

Appellant's position and the teachings of Lobb et al. directly rebut the rejection's position that Lobb et al. provide any teaching or motivation to make the combination with Schramm et al., Wigzell et al., and/or Krause et al. The rejection's position is clearly not a reasonable

conclusion based on the Lobb et al. disclosure and would not be the conclusion of one of skill in the art with the reference of Lobb et al. before him.

Appellant further submits that the VLA-4 integrin of Lobb et al. is a completely different cell surface molecule than the recited T cell-specific receptors, such that *even if* one considers binding of such antibody to a T cell, antibodies that bind to VLA-4 will be expected to have different effects on a T cell than antibodies that bind to one of the T cell receptors recited in the instant claims. For example, anti-VLA4, as taught by Lobb et al. and discussed above, can reduce recruitment of cells to a tissue. In contrast, the recited T cell receptor antibodies are directed against T cell activation receptors and coreceptors, and are depleting or inactivating antibodies (*e.g.,* blocking antibodies are not included). However, the rejection appears to base motivation for combining Lobb et al., Schramm et al., Krause et al. and Wigzell et al. at least in part on the argument that if one antibody that binds to T cells reduces airway hyperresponsiveness, then all antibodies that bind to T cells will reduce airway hyperresponsiveness, regardless of the target protein or mechanism of action, and further, that results with aerosolized antibodies can necessarily be extrapolated from results using systemically administered antibodies, and *vice versa*.

Using this rationale, one of skill in the art should therefore expect that an antibody that binds to LFA-3 would reduce airway hyperresponsiveness, since LFA-3 is another adhesion protein, indeed a protein of similar function to VLA4, that is known in the art to be expressed on T cells, among other cells. However, Lobb et al. demonstrates that anti-LFA3 did not reduce airway hyperreponsiveness when administered to an animal. Referring to Example 2 of Lobb et al. (col. 12, line 37 to col. 13, line 7), where aerosolized anti-VLA-4 administration (antibody

HP1/2) was compared to aerosolized anti-LFA-3 administration (antibody IE6), Lobb et al. clearly teaches that administration of aerosolized anti-LFA-3 had **no effect** on airway hyperresponsiveness.

Therefore, the argument that one could simply substitute a different antibody into the specific anti-VLA4 method of Lobb et al. is not correct and is clearly rebutted in the references of record. Such an argument lacks common sense in view of the clear teaching away from that position in Lobb et al. It is clear from this example alone that the combination of elements inferred by the rejection would not be expected to yield predictable results.

Furthermore, Appellant submits that the previously submitted publication by Fahy et al. (enclosed in Evidence Appendix) rebuts the rejection's line of reasoning that it would be expected that a systemically delivered antibody of Schramm et al. would be useful in the method of aerosolized delivery of Lobb et al., because Fahy et al. shows that provision of a therapeutic effect by administration of antibodies systemically does not necessarily mean that the same effect will be provided when the same antibody is administered by aerosol. As discussed on page 5, lines 10-16, Fahy et al. used aerosolized anti-IgE to test whether direct delivery of the antibody to the airway would have the same effect as the systemic delivery of the antibody, which had already been shown to attenuate early and late phase responses to inhaled allergen (Fahy et al., 1999, *Am. J. Respir. Crit. Care Med.* **160**:1023-1027). Fahy's experiment demonstrated that the aerosolized anti-IgE did not attenuate the airway responses to inhaled allergen and in at least one subject, the antibody proved to be immunogenic. Therefore, this experiment shows that, based on the art, one cannot assume that achievement of a therapeutic effect by administration of an antibody systemically can be extrapolated to aerosol

administration of the same antibody. The argument can be taken a step further in that the rejection has attempted in the present combination of references to compare two *completely different* antibodies on this basis (*i.e.,* antibodies with different antigen specificities), which makes prediction of effects even more unreasonable. Therefore, the rejection's argument that one can take the results of Lobb et al. and thereby predict a result using the antibody of Schramm et al. is not fairly based on scientific evidence. Moreover, the teachings of Wigzell et al. and Krause et al. do not contradict the findings of Fahy et al., as neither of Wigzell et al. or Krause et al. had any actual demonstration of the administration of an antibody in aerosol form (discussed in more detail below).

The rejection reasons on page 9 of the April 6 Office Action that: "Fahy et al. hypothesize that the antibody might have been more immunogenic via the aerosol route, but the successful results of Lobb et al. would tend to disagree with this hypothesis", which is speculation put forth by the rejection itself that is not supported by evidence. As discussed above, Lobb et al. investigated the effects of an antibody with a completely different antigen specificity (VLA-4 versus IgE) on airway hyperresponsiveness and provided results that are in contrast to Fahy et al., illustrating the unpredictability of aerosol administration of antibody. The rejection further reasons on page 9 of the April 6 Office Action that the most logical explanation for the results in Fahy et al. "is that their antibody was not effective was because it was antibody that bound a soluble antigen (IgE) present in large quantities in the vascular space wherein said IgE acted as a 'sink of IgE'", and concludes that the results of Fahy et al. are not germane to the claimed invention. Appellants do not see how Lobb et al., directed to a different antigen specificity than that claimed is relevant to the claimed invention, while Fahy et al. is somehow

not germane to the invention. Moreover, as discussed above, Lobb et al. teach that aerosol delivery of LFA-3, a protein of the same general type as VLA-4, did not inhibit airway hyperresponsiveness. Based on the reasoning in the rejection, including the rationale for the dismissal of Fahy et al. as being irrelevant, the aerosol administration of LFA-3 would have been predicted to inhibit airway hyperresponsiveness; however, it did not.

Given that Appellant finds no basis in Lobb et al. by way of a teaching, suggestion or motivation for making the combination with Schramm et al. and/or the other cited references, Appellant now reviews the rejection's contention that Schramm et al. teach that anti-TCRαβ can be used to treat asthma, which is the rejection's stated basis for the combination of Schramm et al. with Lobb et al. Appellant submits that Schramm et al. do not teach that anti-TCRαβ can be or should be used *to treat asthma*, and more particularly, Appellant submits that Schramm et al. do not teach that *any antibody*, including anti-TCRαβ, can be used to reduce *airway hyperresponsiveness*, which is the subject of the instant claims. As defined in the present specification on page 14, lines 7-9, "'airway hyperresponsiveness' or 'AHR' refers to an abnormality of the airways that allows them to narrow too easily and/or too much in response to a stimulus capable of inducing airflow limitation". Appellant emphasizes that this is the subject of the claimed invention (*i.e.,* the reduction of airway hyperresponsiveness in a mammal). In contrast, the only teaching of Schramm et al. related to antibodies is a teaching that the *systemic depletion* in an animal of αβ T cells using anti-TCRαβ, or the *systemic depletion* of γδ T cells using anti-γδ, significantly reduces eosinophils, and to a lesser extent, lymphocytes and macrophages, in bronchoalveolar lavage fluid (BALF) (see Figure 1). These are the *only* experiments in Schramm et al. that use antibodies; the remaining experiments are performed in

*TCR knockout mice*. With respect to airway hyperresponsiveness, which is the subject of the instant claims, Schramm et al. do not teach that *any* antibody administered by *any* route can reduce airway hyperresponsiveness, nor do Schramm et al. determine the effect of complete αβTCR depletion on airway hyperresponsiveness using the knockout mice. The only experiments directed to airway hyperresponsiveness in Schramm et al. use wild-type mice or TCR δ-/- mice (*i.e.,* TCR δ knockout mice). Schramm et al. specifically state on page 222, col. 2, last sentence of top paragraph "Methacholine responses were not studied in TCRβ -/- mice...".

Moreover, it is Appellant's position that Schramm et al. lacks any teaching or suggestion to use any antibodies for the treatment of asthma. Schramm et al. teaches that anti-αβTCR, administered *systemically*, reduces the accumulation of various cells in BALF, but it is Appellant's position that this is not a teaching that such an antibody could or should be used to treat asthma. Schramm et al. is a research publication that is primarily directed to determining the role of γδ T cells in asthma, and also to dissect the roles of the two T cell subsets (αβ and γδ). Appellant submits that Schramm et al. do not teach or suggest the therapeutic use of *any* antibodies for the treatment of asthma. In general, Appellant does not find any teaching in Schramm et al. regarding how asthma should be treated. Indeed, complete, systemic depletion of T cells, or substantial depletion of T cells, which is the only use of the antibodies described in Schramm et al., would *not* be viewed by one of skill in the art as a therapeutic approach to treatment of a disease, including airway inflammation and/or hyperresponsiveness, because complete, systemic depletion of a major arm of the immune system as a therapy would have clear, undesirable consequences for the animal.

On page 7 of the April 6 Office Action, the rejection argues that "there is no teaching in Schramm et al. that a complete systemic depletion of an entire T cell subset from an animal is required in the antibody treated animals". First, the only experiments described in Schramm et al. pertain to the use of either TCR knockout mice (*i.e.,* there is a "complete" deletion of T cell subset to which the knockout is directed - see, *e.g.,* page 219, col. 1, first paragraph) and the use of anti-TCR antibodies that deplete the animal of the relevant T cells (see, *e.g.,* page 220, col. 2, last paragraph; "Similar findings were observed in mice depleted of TCRγδ or TCRαβ cells by treatment with monoclonal antibodies (Figure 1)"). Therefore, even if the antibodies do not *completely* deplete the mice of the relevant T cells, clearly, the intent of Schramm et al. is to compare the knockout results to antibody depletion; whether or not complete depletion is achieved via antibody administration. Referring to Figure 1 of Schramm et al., the antibody depletion of T cells is significant, and it is Appellant's position that systemic depletion of T cells, whether complete or substantial, would *not* be viewed by one of skill in the art as a therapeutic approach to treatment of a disease.

Second, and perhaps more relevant to the issue at hand, Appellants refer to the discussion above and again emphasize that *in the antibody-treated animals*, Schramm et al. do not evaluate airway hyperresponsiveness; only eosinophil and other cell accumulation in BALF is evaluated (see Figure 1 of Schramm et al.). Moreover, even *when* airway hyperresponsiveness is evaluated, this is only done in the *γδ TCR knockout mice,* and not in the αβ TCR knockout mice (see page 222 of Schramm et al., "Methacholine responses were not studied in TCRβ-\- mice because every animal studied failed to mount an inflammatory immune response to OVA"). Therefore, there is no teaching in Schramm et al. of the effects of the depletion of T cells having

an αβ TCR (complete or partial), by any means, on airway hyperresponsiveness. Accordingly Schramm et al. simply does not teach the use of any antibody against any T cell protein to inhibit airway hyperresponsiveness.

Moreover, Schramm et al. do not teach or suggest the use of aerosolized antibodies or the administration of antibodies to the lung of an animal. One does not learn from the teachings of Schramm et al. that one could or should therapeutically deplete or inactivate the pulmonary T cells in an animal to treat airway hyperresponsiveness in the animal, and moreover, one can not learn from the teachings of Schramm et al. that one can deplete pulmonary T cells and treat airway hyperresponsiveness without substantially affecting peripheral T cells in the animal. Schramm et al. is not at all concerned with therapeutic approaches to reducing airway hyperresponsiveness, nor to any other T cell-expressed proteins, such as the VLA-4 integrin of Lobb et al. Thus, there is no teaching, suggestion, motivation or expectation of success provided by Schramm et al. to make or use the present invention, even when combined with Lobb et al., alone or in combination with the other references. Accordingly, it is submitted that the rejection's basis for the combination of Schramm et al. with Lobb et al. fails, since Appellant finds no teaching or suggestion in Schramm et al. teach that anti-TCRαβ, including aerosolized anti-TCRαβ, nor anti-TCRγδ, including aerosolized anti-TCRγδ, should be used to treat asthma or specifically, to reduce airway hyperresponsiveness.

Having provided arguments against the rejection's reasoning for combining Lobb et al. and Schramm et al., Appellant now addresses the rejection's stated rationale for the inclusion of Wigzell et al. and Krause et al. in the combination.

The rejection contends that Wigzell et al. provide motivation to combine Lobb et al. and Schramm et al. by teaching that pathologic T cells found in the lungs can be treated via intrapulmonary administration of anti-TCR antibody. However, it is submitted that Wigzell et al. is unrelated to the teachings of either of Lobb et al. or Schramm et al. and do not provide any motivation to combine these two references. Wigzell et al. characterizes T cells from the lungs of patients with sarcoidosis, which is an inflammatory disease in which granulomas form on various tissues and organs, including the lung, lymph nodes, skin, and eyes (col. 1, lines 18-21). Therefore, sarcoidosis, and its treatment, are not limited to the lungs. Wigzell et al. identify a particular subset of T cell receptors that appear to be increased in patients with sarcoidosis, and suggest making an antibody to this particular T cell receptor to treat this specific disease. Appellant submits that sarcoidosis is not related to asthma or airway hyperresponsiveness, nor is the identification of the particular T cell subset by Wigzell et al. relevant to asthma or airway hyperresponsiveness, and therefore, there is no teaching, suggestion or motivation in Wigzell et al. to that would cause one of skill in the art to include this reference in the cited combination or in particular, to combine Lobb et al. with Schramm et al. The rejection emphasizes that Wigzell et al. teach intrapulmonary administration of TCR antibody as a basis for motivation. However, intrapulmonary administration is listed among a larger group of "known routes" (col. 13, lines 22-25), and there is nothing in this generic teaching that would lead one of skill in the art reading Wigzell et al. to combine the references of Lobb et al. and Schramm et al. Indeed, since Lobb et al. teach aerosolized administration of anti-VLA4, it is not clear how a teaching by Wigzell et al. that antibodies can be administered by intrapulmonary routes provides any information at all, and particularly, with respect to the combination of Lobb et al. with Schramm et al. Moreover, there

is no demonstration of the actual administration *in vivo* of any antibody in any form in Wigzell et al., including by aerosol, and so regardless of whether or not Wigzell et al. list various possible routes of administration for antibodies, one of skill in the art has absolutely no expectation that aerosol administration of the Wigzell et al. antibody to treat *any* disease, including sarcoidosis, would be successful. Appellant has provided examples above of instances in which systemic administration did not equate to pulmonary administration, and in which results with one antibody did not equate to results with a different antibody. It is Appellant's position that Wigzell et al. is devoid of any teaching that would remedy the deficiencies of the combination of Lobb et al. and Schramm et al. as discussed above, even in view of Krause et al. and/or Arrhenius et al.

Similarly, the rejection asserts that Krause et al. teach that antibodies that inhibit T cell activation are preferably administered via pulmonary aerosol, which allegedly provides motivation to combine Lobb et al. and Schramm et al. Krause et al. teaches the identification of a protein associated with actin cytoskeletal reorganization called "Fyb/SLAP", which, as with the VLA4 of Lobb et al., is not T cell-specific (the protein is expressed also by macrophages, platelets, and perhaps other hematopoietic cells). Krause et al. teach that one may produce an antibody that selectively binds to Fyb/SLAP and administer the antibody to an animal to regulate cytoskeletal reorganization in the animal. In teaching administration of the therapeutics of their invention, Krause teach a variety of routes, similar to Wigzell et al, although Krause et al. states a preference for pulmonary aerosol delivery for the Fyb/SLAP antibodies. However, Appellant submits that regulation of cytoskeletal reorganization is not related to asthma or airway hyperresponsiveness, nor is the antibody described by Krause et al. specific for T cells, and

therefore, there is no teaching, suggestion or motivation in Krause et al. to combine this reference with Lobb et al. and/or Schramm et al. and/or Wigzell et al. Furthermore, the antibody of Krause et al. is not T cell-specific and is a blocking antibody (see section 0008, 0104, 0105), which is not an antibody that binds to any of the recited T cell receptors and is not an antibody that depletes or inactivates T cells, further removing this reference from any relevance to the claimed invention, even when combined with the other references. Finally, since Lobb et al. teach aerosolized administration of anti-VLA4, it is not clear how a teaching by Krause et al. that antibodies can be administered by pulmonary aerosol routes provides any information at all, and particularly, with respect to the combination of Lobb et al. with Schramm et al. As with Wigzell et al., Krause et al. provide no actual demonstration of the administration of any antibody in any form, including by aerosol, and so regardless of whether or not Krause et al. teach that one route of administration is intrapulmonary, one of skill in the art has absolutely no expectation that aerosol administration of the Krause et al. antibody to treat *any* disease would be successful. It is Appellant's position that Krause et al. is devoid of any teaching that would remedy the deficiencies of the combination of Lobb et al. and Schramm et al. as discussed above, even in view of Wigzell et al. and/or Arrhenius et al.

As will be recognized, claims cannot be found obvious unless the prior art teaches or suggests making the claimed product or process and that there is a reasonable expectation of success at doing so. *See In re Vaeck*, 20 USPQ2d 1438 (Fed. Cir., 1991) (The teaching or suggestion to make the claimed combination or modification and the reasonable expectation of success must both be found in the prior art).

In summary, Lobb et al. does not teach or suggest that anti-VLA4 reduces airway hyperresponsiveness by acting on T cells, but instead teaches the use of anti-VLA-4 to inhibit migration of neutrophils and eosinophils (without any showing of an effect on T cells, other than an *increase* in lymphocytes), and further, Lobb et al. additionally teaches that aerosol administration of another antibody that can bind to T cells (among other cells), anti-LFA3, has no effect on airway hyperresponsiveness. Accordingly, this reference cannot provide any teaching, suggestion, motivation or expectation of success at using a T cell-specific antibody that is directed to a completely different antigen and that operates by a completely different mechanism for the treatment of asthma or particularly, airway hyperresponsiveness, regardless of the route of administration. Schramm et al. can not remedy the deficiencies of Lobb et al., because Schramm et al. provides absolutely no connection to the anti-integrin VLA4 antibody, and particularly, because Schramm et al. does not provide any teaching, suggestion, or expectation of success of treating asthma or reducing airway hyperresponsiveness using any antibody that binds to T cells delivered by any route, because there is no teaching or suggestion in Schramm et al. of the use of such an antibody for such a purpose. Wigzell et al. and Krause et al. are not directed to asthma or airway hyperresponsiveness at all, Krause et al. does not describe a T cell-specific antibody, and neither reference provides any demonstration that an antibody administered by any route will have any therapeutic effect on any disease. Accordingly, the combination of references fails to provide sufficient teaching, suggestion, motivation, or expectation of success at arriving at the presently claimed invention.

Finally, Appellant submits that the claimed invention provides unexpected and surprising advantages over the prior teachings in the art. First, the claimed method targets pulmonary T cell

populations in the absence of any substantial effect on peripheral T cells, which is a large advantage over methods which target T cell responses systemically, since peripheral immune responses (i.e., immune responses outside the localized area of delivery, such as in the spleen or lymph nodes) are neither substantially stimulated nor substantially inhibited. Systemically administered antibodies will target all T cells including developing T cells, whereas the aerosolized antibodies of the present invention primarily target T cells at the effector stage, *i.e.* functionally differentiated T cells. The rejection asserts that Lobb et al. meet this claim limitation by teaching that the effect can be achieved without detectable blood levels of antibody (referring to col. 12, last paragraph). However, Lobb et al. merely state at this section of the patent that there were no detectable blood levels of the antibody in the aerosol treated animals. Lobb et al. does not actually demonstrate whether or not the antibody had any effect on any T lymphocytes in the animals. As discussed above, the only teaching of Lobb et al. regarding lymphocytes at all is with respect to Figure 4, where it is shown that anti-VLA4 increases total lymphocytes in the BALF. Even if one assumes, *arguendo*, that aerosolized anti-VLA4 did not substantially affect peripheral T cells, it remains Appellant's position that all evidence in Lobb et al. points to a teaching of an affect on neutrophils and eosinophils, and not lymphocytes, such that there is no teaching or suggestion to arrive at the claimed invention. Moreover, to the extent that the rejection maintains the position that the teachings of Schramm et al. with respect to the systemic administration of antibodies is relevant to the combination of references, it is clear that Schramm et al. do not teach or suggest any advantage of aerosolized antibodies and solely contemplate total systemic depletion of T cells.

Furthermore, in contrast to reports of the administration of other aerosolized antibodies (e.g., anti-IgE administration, described by Fahy et al. (1999, *Am. J. Respir. Crit. Care Med.* **160**:1023-1027)), the present inventors have demonstrated through working examples that the claimed method is highly effective at reducing airway hyperresponsiveness. Finally, evidence has been provided in the present specification that targeting T cells that are present at the allergic site by the localized administration method of the present invention reduces allergic inflammation-associated exacerbation of AHR without affecting the adaptive immune system.

It is noted that Claim 1 is currently restricted to the elected species of αβ TCR, although it is Appellant's position that the non-elected species of γδ TCR, CD3, CD4 and CD8 are also patentable over the cited combination of references, should the rejection be applied to other species, for substantially the same reasons provided herein. With respect to γδ TCR, although Schramm et al. also describe anti-γδ TCR, the teachings of Schramm et al. are thoroughly discussed above, and the consideration of anti-γδ TCR does not render Schramm et al. any more relevant to the combination than when anti-αβ TCR is considered alone, since the deficiencies of Schramm et al., as well as the other references, remain the same. Furthermore, is noted that none of the cited references teach or suggest an antibody that binds to CD3, CD4 or CD8.

However, to be clear, Appellant asserts that the restricted species as set forth by the rejection with respect to αβ TCR, γδ TCR, CD3, CD4 and CD8, do not stand or fall together.

In summary, in view of the discussion above, the combination of references fails to teach or suggest the use of aerosolized antibody that binds to and depletes or inactivates the recited T cells receptors, whereby aerosolized administration of said antibodies reduces airway hyperresponsiveness in a mammal in the absence of substantially stimulating or inhibiting

peripheral T cell responses. Moreover, the combination fails to provide any motivation to make the combination as the rejection has done, or to motivate one to make and use the present invention. Finally, the combination does not provide any expectation of success at making and using the present invention. Therefore, the rejection has not established a *prima facie* case of obviousness in view of the combination of references. In view of the above arguments, Appellant respectfully requests the Board to direct the withdrawal of the rejection of Claims 1, 9-15, 17-18, and 24-35 under 35 U.S.C. §103(a).

**Claim 2**

With respect to Claim 2, Appellant notes that this claim is directed to the elected species of αβTCR, whereas Claim 1 in the group of claims above is directed to all species, including non-elected species that were previously rejoined in the Office Action mailed September 8, 2005, and then subsequently restricted again in the Office Action mailed February 22, 2006. Appellant's arguments against the rejection of Claim 2 under 35 U.S.C. § 103(a) are essentially the same as the arguments presented above in view of Claim 1, although such arguments are in the case of Claim 2 directed exclusively to the elected species. However, in the event that Claim 1 falls as a result of consideration of non-elected species in Claim 1, Appellant expressly submits that Claim 2, as well as dependent Claims 9-15, 17-18, and 24-35, to the extent they depend from Claim 1 with respect to the elected invention of αβTCR as recited in Claim 2, do not stand or fall together with Claim 1.

In view of the above arguments, Appellant respectfully requests the Board to direct the withdrawal of the rejection of Claim 2 under 35 U.S.C. §103(a).

**Claim 16 and Claims 19-23**

In addition to the arguments set forth above for Claims 1, 9-15, 17-18, and 24-35, it is Appellant's further contention that Claims 16 and Claims 19-23 recite particular features of the claimed invention related to advantages of the invention that are not taught or suggested by the combination of references. In particular, Claims 16 and Claims 19-23 recite the use of very low doses of antibody by aerosol administration, which are not taught or suggested by the combination or references, and which it is submitted would be considered to be surprising at the time of the invention. Claim 16 provides the limitation that the antibody is administered at a dose of between about 5 μg antibody and about 10 μg antibody per milliliter of formulation. Claims 19-23 recite doses of less than 40 μg antibody per kg body weight of the mammal (Claim 19), or less than 1 μg per kg body weight of the mammal (Claim 20), or less than 0.5 μg per kg body weight of the mammal (Claim 21), or less than 0.1 μg per kg body weight of the mammal (Claim 22), or less than 20 ng per kg body weight of the mammal (Claim 23).

As taught in the specification, prior to the present invention, it was thought that antibodies delivered by aerosol must be administered in high doses to overcome the effects of expected low potency and to successfully reach the target airways (see page 10, lines 22-26). For example, U.S. Patent 6,165,463 (see Evidence Appendix) indicates that antibodies are considered to be "low potency" drugs, and therefore indicates that fairly high concentrations of antibodies (e.g., in the milligram per milliliter range) should be formulated for aerosol delivery. The publication of Fahy et al. has been discussed above. Indeed, the lowest dose of antibody specifically taught by Lobb et al. (see col. 6, lines 58-62) is 50 μg per kg body weight of the mammal. Lobb et al. also teach that one could provide a dose to "maintain a plasma level of antibody in the range from 1-1000 μg/ml" (col. 6, lines 52-54), but does not state what actual

doses will achieve this range. It is noted that the rejection contends that Lobb et al. specifically teach the dose of Claim 19 (less than 40μg per kg body weight) in col. 6, but such teaching is not found by Appellant. Lobb et al. also teach that the effect of anti-VLA4 (HP1/2) was dose-dependent, and that with intravenous administration, the dose was ineffective below 0.2 mg/kg (col. 12, lines 25-28). Given the teachings in the art at the time of the invention, one would therefore assume that an even higher dose would be needed if the antibody was delivered by aerosol. Delivery of aerosolized antibody in Lobb et al. was provided at 8mg per sheep. Even assuming a 100 kg sheep, this would still be 80 μg per kg body weight. With regard to Schramm et al., this reference does not teach aerosol administration of antibody. With regard to Wigzell et al. and Krause et al., neither of these references demonstrates the delivery of any antibody by any route of administration and provides no specific direction regarding doses for *aerosol* administration. Given the teachings in the art at the time of the invention, such as Fahy et al., it is submitted that one of skill in the art would not expect efficacy in delivering an antibody at the low doses claimed in Claims 16 and 19-23.

In contrast, the method of the present invention is effective at extremely *low* doses of antibody. Indeed, the method of the present invention achieves efficacy with antibody doses that are believed to be about *1000-fold* or more lower than systemic doses of antibody required to achieve the same effect. Doses of antibody as low as 5 μg per ml, delivered by nebulizer to mice in a plexiglass chamber, which would actually deliver much smaller doses to the airway of each mouse, were effective at reducing airway hyperresponsiveness.

The rejection reasons that with respect to the particular recited dosages of formulation or dosage per weight, a "routineer" would initially test a wide variety of different dosages in order

to determine the smallest effective dosage. However, as discussed above, at the time of the invention, it was not generally thought that aerosol delivery of antibodies was efficient or could be achieved at very low doses.

In view of the above arguments, Appellant respectfully requests the Board to direct the withdrawal of the rejection of Claims 16 and 19-23 under 35 U.S.C. §103(a).

**Claim 36**

In addition to the arguments set forth above for Claims 1, 9-15, 17-18, and 24-35, it is Appellant's further contention that the cited combination of combination of references fails to teach or suggest the use of an aerosolized antibody having one of the particularly recited receptor specificities to reduce airway hyperresponsiveness, wherein the binding of the antibody to the receptor causes the depletion or inactivation of the T cell and *wherein any stimulation or inhibition of peripheral T cell responses in the mammal after the aerosolized antibody administration is less than about 10% of stimulation or inhibition of the peripheral T cell responses that would be detected if the antibody formulation was administered systemically*, as claimed in Claim 36. The claimed method targets pulmonary T cell populations in the absence of any substantial effect on peripheral T cells, which is a large advantage over methods that target T cell responses systemically, since peripheral immune responses (i.e., immune responses outside the localized area of delivery, such as in the spleen or lymph nodes) are neither substantially stimulated nor substantially inhibited. Systemically administered antibodies will target all T cells including developing T cells, whereas the aerosolized antibodies of the present invention primarily target T cells at the effector stage, *i.e.* functionally differentiated T cells. The specification demonstrates that aerosolized administration of the recited antibody reduces

airway hyperresponsiveness in a mammal within the claim limitations of Claim 36 (*e.g.,* see Example 5, where any stimulation or inhibition of peripheral T cell responses in the mammal after the aerosolized antibody administration is less than about 10% of stimulation or inhibition of the peripheral T cell responses that would be detected if the antibody formulation was administered systemically).

Appellant refers to the arguments set forth in detail with respect to 1, 9-15, 17-18, and 24-35, and further submit that none of the cited references, alone or in combination, teach or suggest the claimed limitation that any stimulation or inhibition of peripheral T cell responses in the mammal after the aerosolized antibody administration is less than about 10% of stimulation or inhibition of the peripheral T cell responses that would be detected if the antibody formulation was administered systemically. The rejection reasons that Lobb et al. teach that "the effect seen can be achieved without detectable blood levels of antibody...wherein the aerosol administered antibody would therefore not substantially effect peripheral immune T cell responses", referring to col. 12, last paragraph).

However, it is Appellant's position that Lobb et al. merely state in their patent that there were no detectable blood levels of the antibody (*i.e.,* referenced as "the drug") in the aerosol treated animals. Lobb et al. does not actually demonstrate whether or not the antibody had any effect on any T lymphocytes or any other cells in the periphery of the animals, and specifically, Lobb et al. does not teach that stimulation or inhibition of peripheral T cell responses in the mammal after the aerosolized antibody administration is less than about 10% of stimulation or inhibition of the peripheral T cell responses that would be detected if the antibody formulation was administered systemically. Lobb et al. does not perform an experiment to evaluate T cells,

other than to determine that lymphocyte numbers increase in BALF after anti-VLA4 treatment, and has very little disclosure related to T cells at all, and so one can not conclude that Lobb et al. teach the subject matter of Claim 36. Similarly, no such information or evidence is provided in the teachings of Wigzell et al. or Krause et al., and Schramm et al. solely discloses systemic depletion of T cells. Accordingly, it is submitted that the combination of references fails to teach the limitation disclosed in Claim 36, and furthermore, fails to provide any motivation to modify any of the teachings to arrive at the claimed invention.

In view of the above arguments, Appellant respectfully requests the Board to direct the withdrawal of the rejection of Claim 36 under 35 U.S.C. §103(a).

## VIII. CLAIMS APPENDIX

The text of the claims involved in this appeal:

1. A method to reduce airway hyperresponsiveness in a mammal that has, or is at risk of developing, airway hyperresponsiveness, comprising administering to the lungs of said mammal an aerosolized antibody formulation comprising antibodies that selectively bind to a receptor on a T cell selected from the group consisting of: a T cell antigen receptor (TCR) selected from the group consisting of an αβ TCR and a γδ TCR, CD3, CD4 and CD8, wherein the binding of the antibodies to the receptor causes the depletion or inactivation of the T cell, wherein administration of the antibody formulation reduces airway hyperresponsiveness in said mammal; and

wherein the administration of the aerosolized antibody formulation affects pulmonary T cell responses in the mammal, while peripheral T cell responses in the mammal are neither substantially stimulated nor substantially inhibited.

2. The method of Claim 1, wherein said receptor on a T cell is an αβ T cell antigen receptor (TCR).

9. The method of Claim 1, wherein said antibody is a humanized monoclonal antibody.

10. The method of Claim 1, wherein said antibody does not stimulate T cell activation.

11. The method of Claim 1, wherein said antibody is a monovalent antibody.

12. The method of Claim 1, wherein said antibody is a neutralizing antibody.

13. The method of Claim 1, wherein said aerosolized antibody formulation is administered at a dose of less than about 500 μg antibody per milliliter of formulation.

14. The method of Claim 1, wherein said aerosolized antibody formulation is administered at a dose of less than about 100 μg antibody per milliliter of formulation.

15. The method of Claim 1, wherein said aerosolized antibody formulation is administered at a dose of less than about 50 μg antibody per milliliter of formulation.

16. The method of Claim 1, wherein said aerosolized antibody formulation is administered at a dose of between about 5 μg antibody and about 10 μg antibody per milliliter of formulation.

17. The method of Claim 1, wherein said aerosolized antibody formulation comprises less than 35% by weight of said antibody.

18. The method of Claim 1, wherein said aerosolized antibody formulation is administered at a dose of less than about 400 μg x kilogram$^{-1}$ body weight of said mammal.

19. The method of Claim 1, wherein said aerosolized antibody formulation is administered at a dose of less than about 40 μg x kilogram$^{-1}$ body weight of said mammal.

20. The method of Claim 1, wherein said aerosolized antibody formulation is administered at a dose of less than about 1 μg x kilogram$^{-1}$ body weight of said mammal.

21. The method of Claim 1, wherein said aerosolized antibody formulation is administered at a dose of less than about 0.5 μg x kilogram$^{-1}$ body weight of said mammal.

22. The method of Claim 1, wherein said aerosolized antibody formulation is administered at a dose of less than about 0.1 μg x kilogram$^{-1}$ body weight of said mammal.

23. The method of Claim 1, wherein said aerosolized antibody formulation is administered at a dose of less than about 20 ng x kilogram$^{-1}$ body weight of said mammal.

24. The method of Claim 1, wherein said aerosolized antibody formulation comprises a pharmaceutically acceptable carrier.

25. The method of Claim 24, wherein said pharmaceutically acceptable carrier is selected from the group consisting of: a dry, dispersible powder; small capsules; liposomes; and a nebulized spray.

26. The method of Claim 1, wherein said aerosolized antibody formulation is

administered to said mammal in conjunction with another agent that supports the treatment of AHR selected from the group consisting of: corticosteroids, (oral, inhaled and injected), â-agonists (long or short acting), leukotriene modifiers (inhibitors or receptor antagonists), antihistamines, phosphodiesterase inhibitors, sodium cromoglycate, nedocrimal, and theophylline.

27. The method of Claim 1, wherein said mammal has been sensitized to an allergen and has been exposed to, or is at risk of being exposed to, an amount of said allergen that is sufficient to induce airway hyperresponsiveness (AHR) in said mammal in the absence of said aerosolized antibody formulation.

28. The method of Claim 1, wherein said aerosolized antibody formulation is administered within a time period of between 48 hours or less prior to exposure to an AHR provoking stimulus that is sufficient to induce AHR, and within 48 hours or less after the detection of the first symptoms of AHR.

29. The method of Claim 1, wherein said aerosolized antibody formulation is administered upon the detection of the first symptoms of acute onset AHR.

30. The method of Claim 1, wherein said aerosolized antibody formulation is administered within 1 hour after the detection of the first symptoms of acute onset AHR.

31. The method of Claim 1, wherein said aerosolized antibody formulation is administered within 12 hours or less prior to exposure to a AHR provoking stimulus that is sufficient to induce acute onset AHR.

32. The method of Claim 1, wherein said aerosolized antibody formulation is administered within 2 hours or less prior to exposure to a AHR provoking stimulus that is sufficient to induce acute onset AHR.

33. The method of Claim 1, wherein administration of said aerosolized antibody formulation reduces the airway hyperresponsiveness of said mammal such that the $FEV_1$ value of

said mammal is improved by at least about 5%.

34. The method of Claim 1, wherein said mammal is a human.

35. A method to reduce airway hyperresponsiveness in a mammal that has, or is at risk of developing, airway hyperresponsiveness, comprising administering to the lungs of said mammal an aerosolized antibody formulation comprising antibodies that selectively bind to a receptor on a T cell selected from the group consisting of: a T cell antigen receptor (TCR) selected from the group consisting of an αβ TCR and a γδ TCR, CD3, CD4 and CD8, wherein the binding of the antibodies to the receptor causes the depletion or inactivation of the T cell, wherein administration of the antibody formulation reduces airway hyperresponsiveness in said mammal; and

wherein any stimulation or inhibition of peripheral T cell responses in the mammal after the aerosolized antibody administration is less than about 10% of stimulation or inhibition of the peripheral T cell responses that would be detected if the antibody formulation was administered systemically.

## IX. EVIDENCE APPENDIX

A. Fahy et al., 1999, *Am. J. Respir. Crit. Care Med.* **160**:1023-1027 (Submitted in PTO-1449 filed October 4, 2001, and considered by the Examiner on July 17, 2003; cited as evidence by Applicants in Amendment and Response filed May 13, 2003)

B. U.S. Patent No. 6,165,463 (Submitted in PTO-1449 filed October 4, 2001, and considered by the Examiner on July 17, 2003; cited as evidence by Applicants in Amendment and Response filed October 14, 2003).

C. U.S. Patent No. 6,171,799 (Submitted in PTO-1449 filed October 4, 2001, and considered by the Examiner on July 17, 2003; discussed by Applicants in the Specification at page 3).

## X. RELATED PROCEEDINGS APPENDIX

None.

## XI. SIGNATURE OF APPELLANT'S REPRESENTATIVE

Correspondence related to this Appeal Brief should be directed to the undersigned, who may also be contacted at (303) 863-9700.

Respectfully submitted,

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Date: November 3, 2008

# Effect of Aerosolized Anti-IgE (E25) on Airway Responses to Inhaled Allergen in Asthmatic Subjects

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**Intravenous administration of a humanized monoclonal antibody of IgE (E25) attenuates the early and late phase response to inhaled allergen in allergic asthmatic subjects. To test whether direct delivery of E25 to the airway might have the same effect, we conducted a randomized, double-blind, three group study in 33 subjects with mild allergic asthma (20 to 46 yr of age, 21 men, $FEV_1 > 70\%$ predicted). The airway responses to aerosolized allergen were determined at baseline, after 2 and 8 wk of once daily treatment with aerosolized placebo (n = 11), aerosolized E25 1 mg (n = 12), or aerosolized E25 10 mg (n = 10), and after 4 wk of treatment withdrawal. We found that E25 was detectable in the serum during aerosol treatment, although serum IgE did not change significantly in any of the three groups during treatment. In addition, both doses of E25 were no more effective than placebo in attenuating the early phase responses to allergen at both times during treatment. Although aerosolized E25 was generally well tolerated, one subject receiving aerosolized E25 10 mg daily was found to have serum IgG and IgA antibodies to E25. We conclude that aerosol administration of an anti-IgE monoclonal antibody does not inhibit the airway responses to inhaled allergen in allergic asthmatic subjects. We speculate that the observed lack of efficacy may be due to the inability of aerosol route of delivery to result in high enough concentrations of E25 in the tissue compartments surrounding IgE effector cells to neutralize IgE arising from local airway and pulmonary sources and IgE arising from the vascular space. Additionally, the aerosol route of delivery of monoclonal antibodies may be more immunogenic than the parenteral route. Fahy JV, Cockcroft DW, Boulet L-P, Wong HH, Deschesnes F, Davis EE, Ruppel J, Su JQ, Adelman DC. Effect of aerosolized anti-IgE (E25) on airway responses to inhaled allergen in asthmatic subjects.**

AM J RESPIR CRIT CARE MED 1999;160:1023–1027.

Recombinant humanized monoclonal antibody-E25 or "E25" is a nonanaphylactogenic anti-IgE antibody that attenuates both the early and late phase responses to inhaled allergen in asthmatic subjects (1, 2). In this study, we examined whether aerosolized R25 attenuates the airway responses to inhaled allergen in allergic asthmatic subjects. To do this, we conducted a randomized, placebo-controlled, parallel group clinical trial of the effects of 8 wk of once daily treatment with aerosolized E25 in two doses (1 mg and 10 mg) on the early and late phase responses to allergen challenge in allergic subjects with mild asthma.

Thirty-three subjects with asthma with $FEV_1 \geq 70\%$ predicted, bronchial hyperactivity to methacholine, serum IgE < 500 IU/mL, a positive skin prick test to aeroallergens (house dust mite, perennial ryegrass, birch, cat pelt, or horse hair) were studied (Table 1 and Figure 1). Exclusion criteria were the use of any corticosteroids or symptoms of an upper or lower respiratory tract infection in the previous 6 wk, and history of tobacco use (any in the past 12 mo and total use ≥ 10 pack-years). The study protocol and consent form was approved by the committees for human research at each participating institution, and each subject provided written informed consent.

Subjects were randomized to E25-1 mg, E25-10 mg, or matching placebo (E25 excipient [150 mM NaCl, 10 mM acetate at pH 5.2]); randomization was stratified according to whether the subject had a late phase response to allergen during the screening phase. Subjects self-administered medication once daily at home using a PARI IS-2 nebulizer powered by a PARI Master compressor. On the basis of data from aerosol experiments in monkeys (3), the E25-1 mg dose was predicted to deliver 150 μg to the lower airways and the E25-10 mg dose was predicted to deliver 1.5 mg.

Skin reactivity to house dust mite (*Dermatophagoides pter-*

(*Received in original form October 5, 1998 and in revised form April 5, 1999*)

Supported by a research grant from Genentech Inc.

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TABLE 1

CLINICAL CHARACTERISTICS OF THE STUDY SUBJECTS*

| Characteristics | Placebo (*n* = 9) | E25-1 mg (*n* = 12) | E25-10 mg (*n* = 10) |
|---|---|---|---|
| Age, yr | 28 ± 8 | 28 ± 8 | 30 |
| Weight, kg | 72 ± 16 | 73 ± 20 | 88 ± 21 |
| $FEV_1$, % pred | 81 ± 14 | 83 ± 20 | 84 ± 14 |
| $PC_{20}$, mg/ml† | 0.8 | 1.4 | 1.1 |
| | (0.3–2.4) | (0.7–2.6) | (0.5–2.5) |
| IgE, IU/L | 208 ± 171 | 250 ± 141 | 226 ± 153 |

* Plus-minus values are means ± SD.

† $PC_{20}$ denotes the concentration of methacholine that causes a 20% fall in $FEV_1$. Values are presented as the geometric mean (95% CI).

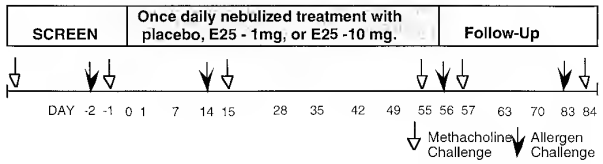


***Figure 1.*** Diagrammatic representation of the three-phrase, 14- to 16-wk, randomized, double-blind, placebo-controlled, parallel group trial. There was a 2- to 4-wk screening phase, followed by an 8-wk treatment phase, followed by a 4-wk follow-up phase. During the screening phase, subjects were characterized by spirometry, methacholine reactivity, and allergen skin tests, and they were taught to record their peak flows in a diary using a Mini-Wright Peak Flow Meter (Clement Clarke, Columbus, OH). Subjects then underwent baseline methacholine challenge and airway allergen challenge in the sequence depicted in the diagram, after which they began self-administering study drug (E25-1 mg, E25-10 mg, or matched placebo) once a day by nebulizer for 8 wk. Methacholine challenge and allergen challenge were repeated as shown in the diagram. Spirometry was performed on Days 0, 14, 28, 42, 56, 70, and 84. Samples of venous blood for standard tests of hematologic, renal, and hepatic function were collected once during the screening phase and again on Days 0, 14, 28, 56, and 83. Samples of venous blood for levels of free IgE, total IgE, and rhuMAb-E25 were collected at Days 0, 14, 28, 42, 56, 70, and 83. Finally, bronchoscopy and bronchoalveolar lavage was performed on the 10 subjects enrolled at the Québec center during the screening phase and again on Day 42.

*onynisinus* and *Dermatophagoides farinae*), cat pelt, ryegrass (*Lolium perenne*), birch (*Betula* spp), and histamine (1.8 mg/ml), all from Bayer Pharmaceuticals (Spokane, WA) was assessed, as previously described (1, 2).

Bronchial reactivity to methacholine (4) and allergen (1) was determined as previously described. Allergen challenges during the treatment and follow-up phases were performed similarly to baseline, except that the first allergen concentration was two doubling doses below the allergen concentration causing a 20% fall in $FEV_1$ at baseline. During the treatment and follow-up phases, the allergen challenge continued until the $FEV_1$ fell by ≥ 20% or until the same allergen concentration given at baseline was delivered, whichever occurred first.

Total IgE in serum was measured using a microparticle enzyme immunoassay (Abbott Laboratories, Abbott Park, IL). Total IgE in BAL was measured using a more sensitive ELISA (lower limit of detection, 10 pg/ml) as follows: 96-well plates coated overnight with 100 ng of monoclonal anti-IgE antibody in carbonate buffer at pH 9.6 were washed and 100 μl of sample were added. The captured IgE was detected with goat anti-human IgE-biotin (Kirkegaard and Perry, Gaithersburg, MD) and streptavidin-β-galactosidase (Boehringer Mannheim, Indianapolis, IN) followed by 4-methylumbelliferyl-β-D-galactoside substrate (Sigma Chemical Co., St. Louis, MO). The reaction was stopped with 0.3 M glycine at pH 10.5. The fluorescence was read using 360 nm excitation and 460 nm emission wavelength. Free IgE (IgE not in a complex with E25), total E25, free E25, and IgG anti-E25 Fab antibody were measured in blood by ELISA, as previously described (1). Total E25 in serum and BAL samples was measured by ELISA as previously described (1). IgA and IgM class anti-E25 antibodies in blood were assayed similarly using plates coated overnight at 4°C with 300 ng of E25 Fab fragment in 100 μl of PBS. The plates were washed with 0.05% Tween 20 in PBS, then incubated with assay diluent (0.5% BSA/0.05% Tween 20/0.01% thimerosal in PBS) for 1 h to block nonspecific binding sites. Samples were diluted 1/100 in assay diluent, either with added excipient control or with 100 μg/ml E25. The plates were washed and the diluted samples were incubated in the wells for 1 h. The plates were washed and either goat anti-

TABLE 2

PULMONARY FUNCTION AT BASELINE, AT THE END OF TREATMENT, AND DURING FOLLOW-UP*

| Outcome | Study Group | Day 0 | Day 14 | Day 56 | Day 83 |
|---|---|---|---|---|---|
| $FEV_1$, L | Placebo (n = 9) | 3.1 ± 0.6 | 3.1 ± 0.6 | 3.2 ± 0.6 | 3.2 ± 0.6 |
| | E25-1 mg (n = 12) | 3.4 ± 1.1 | 3.6 ± 1.1 | 3.7 ± 1.1 | 3.6 ± 1.1 |
| | E25-10 mg (n = 10) | 3.4 ± 0.9 | 3.5 ± 0.9 | 3.8 ± 0.8 | 3.6 ± 0.9 |
| | Study Group | Week −1 | Week 2 | Week 8 | Week 12 |
| AM PEF, L/min† | Placebo (n = 9) | 456 ± 99 | 460 ± 84 | 452 ± 98 | 463 ± 108 |
| | E25-1 mg (n = 12) | 494 ± 96 | 493 ± 88 | 498 ± 99 | 498 ± 102 |
| | E25-10 mg (n = 10) | 532 ± 78 | 527 ± 79 | 529 ± 75 | 515 ± 89 |
| | Study Group | Day −1 | Day 15 | Day 57 | Day 84 |
| $PC_{20}$, mg/ml | Placebo (n = 8) | 0.95 | 0.77 | 1.17 | 1.0 |
| | | (0.33–2.73) | (0.30–2.00) | (0.38–3.61) | (0.41–2.42) |
| | E25-1 mg (n = 9) | 0.92 | 1.39 | 1.33 | 1.31 |
| | | (0.40–2.14) | (0.61–3.16) | (0.43–4.13) | (0.58–2.98) |
| | E25-10 mg (n = 10) | 1.07 | 0.82 | 1.41 | 1.19 |
| | | (0.48–2.38) | (0.40–1.70) | (0.53–3.74) | (0.55–2.56) |

* Plus-minus values are means ± SD; $PC_{20}$ values are presented as the geometric mean (95% CI).

† AM PEF refers to peak expiratory flow rates measured in the morning before bronchodilator medication was taken.

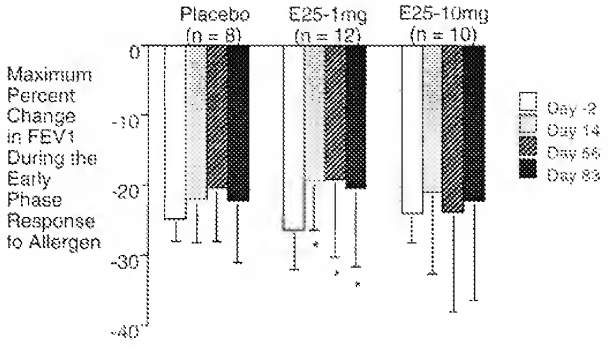


***Figure 2.*** Effect of aerosolized E25 on the change in $FEV_1$ observed during the first hour after allergen challenge (early phase response) in the three treatment groups for each of the four allergen challenges performed during the study (*see* Figure 1). Data are presented as mean ± SD. *Asterisks* denote significantly different from Day −2, but not significantly different from the change seen in the placebo group).

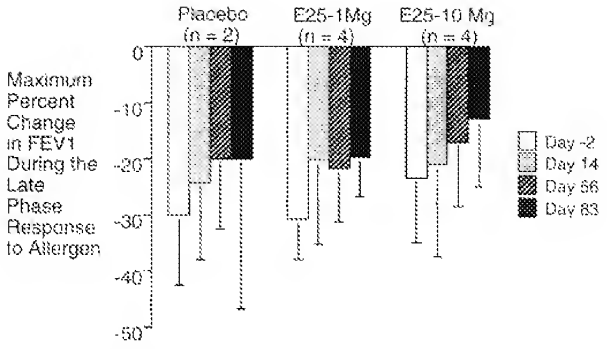


***Figure 3.*** Effect of aerosolized E25 on the change in $FEV_1$ observed between 3 and 7 h after allergen challenge (late asthmatic response) in the subgroup of subjects in each treatment group who demonstrated a significant late phase response during the screening phase. Data are presented as mean ± SD.

human IgA-HRP conjugate (American Qualex) diluted 1/1,000 or goat antihuman IgM-HRP conjugate (Sigma) diluted 1,500 was added. After incubation in the wells for 1 h, the plates were washed and the color was developed using OPD.

Total E25 and IgE concentrations in BAL fluid were corrected for dilution using a urea-based dilution factor. Urea was measured in the BAL using a blood urea nitrogen end point assay kit (Sigma) and a modified protocol for sensitive urea detection (5).

Bronchoscopy was performed as previously described (6). For bronchoalveolar lavage (BAL) three 50-ml boluses of 0.9% saline solution at 37° C were instilled and then aspirated. The BAL fluid was centrifuged at 2 to 8° C for 10 min; 2 ml of the supernatant was aliquoted and diluted with 2 ml of the BAL diluent (1% BSA/0.1% Tween 20/20 mM phosphate/0.9% NaCl). The diluted BAL sample was filtered through a 0.22 μM centrifugal filter (Millipore Ultrafree MC; Millipore Corp., Bedford, MA) at 4,000 × *g* for 5 min.

Data are described as mean and standard deviation or as the geometric mean with 95% confidence intervals, as appropriate. Values for peak flow were analyzed by calculating weekly averages from daily data collections. The area under the curve (AUC) for allergen-induced changes in $FEV_1$ during the early (0 to 1 h) and late (3 to 7 h) phases was calculated using the trapezoidal rule (percent fall in $FEV_1$ × minutes). Between- and within-group comparisons were made using Wilcoxon's rank sum and signed-rank tests, respectively. A p value ≤ 0.05, using two-tailed tests, was considered statistically significant.

Thirty-one of the 33 enrolled subjects completed the study. One subject in the placebo arm developed an asthma exacerbation on Day 7 of treatment, and another subject in the placebo arm withdrew consent on Day 11 because of a change in living location. One additional subject in the placebo group was unable to undergo methacholine on Days 55 and 57 or allergen challenge on Day 56 because of acute labyrinthitis. Overall, aerosolized E25 was well tolerated (Table 2). There were no serious adverse events during the study, and there were no statistically significant differences among treatment groups in the incidence of adverse events. However, whereas only three of 11 subjects in the placebo group reported headache, nine of 12 subjects in the 1 mg group and eight of 10 subjects in the 10 mg group reported headache during the treatment phase.

Compliance was assessed by counting used and unused medication vials at each study visit during the treatment phase. Using this measure of compliance, we found that nine of the 12 subjects in the placebo group, 11 of the 12 subjects in the 1 mg group, and nine of the 10 subjects in the 10 mg group completed at least 90% of all 56 treatments.

Serum total IgE levels (free IgE + IgE complexed to E25) did not change significantly during the treatment phase, and serum-free IgE concentrations were similarly unaffected (data not shown). Changes in concentrations of serum total or free IgE were not expected because of the low serum concentra-

**TABLE 3**

**EFFECTS OF E25 AND PLACEBO ON EARLY AND LATE PHASE AIRWAY RESPONSES ASSESSED AS AREA UNDER THE CURVE FOR CHANGE IN $FEV_1$ DURING ALLERGEN CHALLENGE***

| Outcome | Study Group | Day 0 | Day 14 | Day 56 | Day 83 |
|---|---|---|---|---|---|
| Early response | Placebo (n = 8) | −980 ± 186 | −910 ± 424 | −801 ± 338 | −874 ± 407 |
| | E25-1 mg (n = 12) | −1,058 ± 310 | −767 ± 395 | −783 ± 363 | −842 ± 527 |
| | E25-10 mg (n = 10) | −925 ± 320 | −797 ± 431 | −884 ± 653 | −880 ± 653 |
| Late response | Placebo (n = 2) | −3,675 ± 2,221 | −3,130 ± 959 | −3,000 ± 3,088 | −4,500 ± 8,545 |
| | E25-1 mg (n = 4) | −5,483 ± 752 | −2,948 ± 2,553 | −3,593 ± 2,715 | −2,400 ± 2,100 |
| | E25-10 mg (n = 4) | −3,855 ± 2,172 | −2,835 ± 2,957 | −1,665 ± 1,693 | −1,695 ± 2,405 |

* Plus-minus values are means ± SD. The area under the curve (AUC) for allergen-induced changes in $FEV_1$ during the early (0 to 1 h) and late (3 to 7 h) phases was calculated using the trapezoidal rule (percent fall in $FEV_1$ × minutes).

TABLE 4

NUMBER OF SUBJECTS WITH DETECTABLE SERUM E25 CONCENTRATIONS*

| Group | Day 0 | Day 14 | Day 28 | Day 42 | Day 56 | Day 70 | Day 83 |
|---|---|---|---|---|---|---|---|
| Placebo | 1/11 (28.0) | 0/11 (ND) | 0/11 (ND) | 0/11 (ND) | 0/11 (ND) | 0/11 (ND) | 0/11 (ND) |
| E25-1 mg | 0/12 (ND) | 1/12 (20) | 4/12 (23 ± 5) | 4/12 (33 ± 11) | 3/12 (37 ± 17) | 1/12 (35) | 1/12 (20) |
| E25-10 mg | 0/10 (ND) | 10/10 (73 ± 34) | 8/10 (105 ± 50) | 6/10 (143 ± 71) | 6/10 (143 ± 80) | 4/10 (98 ± 61) | 3/10 (61 ± 39) |

* Values in parentheses represent the mean ± SD E25 level in ng/ml for the subjects in whom a detectable level was found.

tions of E 25. Concentrations of serum E 25 were insufficient to complex significant amounts of serum IgE.

The concentration of allergen delivered during the treatment period was similar to the concentration delivered at baseline in all the treatment groups. For example, in the E 25-10 mg group, the concentration of allergen delivered on Day 56 was 0.10 doubling doses less on average (standard deviation of 0.32 doubling doses) than at baseline; the corresponding doubling dose values for the E 25-1 mg group and the placebo were 0.58 (2.07) and 0.25 (0.71), respectively; $p = 0.78$ between groups. Treatment with E 25-1 mg was associated with a significant within-group attenuation in the early phase response to allergen, but this change was not significantly greater than placebo, and the E 25-10 mg treatment group did not show any significant within- or between-group effect (Figure 2 and Table 3). Two subjects in the placebo group, four subjects in the E 25-1 mg group, and four subjects in the E 25-10 mg group had a late phase response to allergen. In this small subgroup of subjects, there was no statistically significant difference in the magnitude of the late phase response during treatment in any group (Figure 3, Table 3).

E 25 was undetectable in serum samples from the placebo group except for a single observation on Day 0 (Table 4). Serum levels of E 25 were detectable in both the low dose (1 mg) and the high dose (10 mg) groups. In the low dose group, detectable serum levels of E 25 were found in four of the 12 subjects at some point during the study period (Table 4). In the high dose group, a larger production of subjects had detectable serum E 25 levels, especially during the initial treatment period when all 10 of the subjects had detectable levels (Table 4 and Figure 4). Notably, the frequency with which E 25 levels were detectable in the E 25-10 mg group declined during the treatment phase from 10/10 to 6/10 (Table 4 and Figure 4). The number of empty medication vials returned by subjects in the high dose group was similar for all treatment visits.

A serum IgG antibody directed against E 25 was detected at Day 28 of treatment in one subject (Subject 0204) in the high dose E 25 group (Table 5); antibodies were not detected in any other subject in any other group. This finding coincided with a decline in serum E 25 concentration in this subject. The antibody remained detectable at the end of the follow-up period (Day 83), but it was undetectable during an additional special follow-up visit 11 wk after study completion. Clinical examination, chest radiograph, pulmonary function tests (including a test of diffusing capacity), and analysis of blood and urine did not reveal any evidence of immune-complex-mediated disease in this subject 11 wk after study completion.

Four subjects in the 10 mg group had no detectable E 25 in blood at Day 56 (Figure 4). Blood samples from these four subjects were extensively studied for anti-E 25 antibodies of IgG, IgA, and IgM classes. The subject who had an IgG anti-E 25 response also had an IgA anti-E 25 response. No significant reactivity of any antibody class was found in the other three subjects.

The levels of E 25 in BAL were highly variable (Table 6) but generally within the range expected from data available from a preclinical aerosol study in cynomolgus monkeys (Theresa Sweeney, Ph.D, personal communication). Notably, one of the subjects in the 10-mg group had no detectable E 25 in BAL on Day 42. Serum levels of E 25 were also undetectable for this subject from Day 28 onward. No positive anti-E 25, IgG, IgA, or IgM antibody titers were detected in this subject, and there was no indication of noncompliance. The disappear-

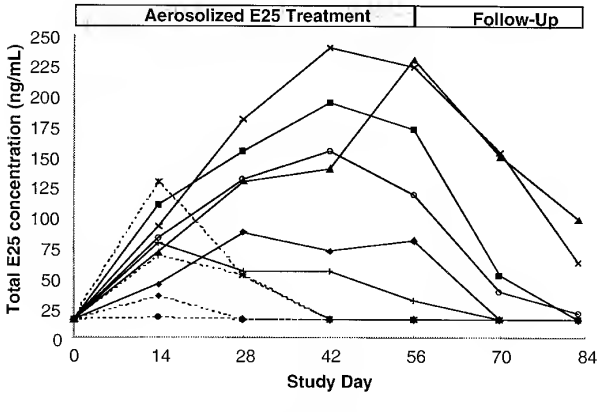


*Figure 4.* E25 levels in blood from the 10 subjects in the 10 mg group at different time points during the 8-wk treatment period and during the 4-wk follow-up period. Four subjects (*dashed lines*) had E25 levels that were undetectable by Day 42 of treatment despite higher levels earlier in the treatment period.

TABLE 5

LEVELS OF E25 AND IgG ANTI-E25 IN SUBJECT 0204 IN THE E25-10 mg GROUP

| Day | Free Serum E25 (*ng/ml*) | Total Serum E25* (*ng/ml*) | Anti-E25 Titer† |
|---|---|---|---|
| 0 | ND | ND | < 2.0 |
| 14 | ND | 35.3 | < 2.0 |
| 28 | ND | ND | 2.1 |
| 42 | ND | ND | 2.4 |
| 56 | ND | ND | 2.5 |
| 70 | ND | ND | 2.4 |
| 83 | ND | ND | 2.4 |
| 158 | Not done | ND | < 2.0 |

* Refers to E25 complexed to IgE (free E25 refers to E25 not in a complex with IgE).
† Refers to anti-Fab titer. Anti-Fc titer was < 2.0 at all time points.
ND denotes not detected.

TABLE 6

LUNG-DEPOSITED DOSES OF E25, BAL IgE LEVELS, AND E25:IgE RATIOS IN THE SUBGROUP OF 10 SUBJECTS WHO UNDERWENT BAL

| Subject No. | Group | Theoretical Deposited Daily Dose ($\mu g$) | Estimated Deposited Dose ($\mu g$) | BAL Dilution Corrected IgE ($\mu g/ml$) | Ratio of BAL E25/BAL IgE |
|---|---|---|---|---|---|
| 1 | Placebo | 0 | 0 | NC | NC |
| 2 | Placebo | 0 | 0 | 0.103 | NC |
| 3 | Placebo | 0 | 0 | 0.131 | NC |
| 4 | Placebo | 0 | 0 | 0.092 | NC |
| 5 | E25-1 mg | 150 | 170–274 | 0.045 | 301 |
| 6 | E25-1 mg | 150 | 19–31 | 0.085 | 18 |
| 7 | E25-1 mg | 150 | 707–1,140 | 0.169 | 336 |
| 8 | E25-10 mg | 1,500 | 1,470–2,380 | 0.059 | 2,000 |
| 9 | E25-10 mg | 1,500 | 273–440 | 0.100 | 221 |
| 10 | E25-10 mg | 1,500 | ND | 0.058 | NC |

*Definition of abbreviations*: NC = not calculated; ND = not detected.

ance of detectable drug levels in the lung and serum may still be due to noncompliance or to anti-E25 antibodies of IgG, IgA, or IgM isotypes not identified by the immunoassays used here. Total IgE concentrations in BAL ranged from 12 to 151 ng/ml and averaged approximately 15% of the serum concentrations of total IgE at screening visit 1 (Table 6). Total IgE concentrations in BAL on Day 42 ranged from 45 to 169 ng/ml and averaged 21% of serum total IgE concentrations. Calculated ratios of E25:IgE from Day 42 BAL samples ranged from 18.3 to 2,000:1 (Table 6).

The main finding of our study is that E25, a nonanaphylactogenic anti-IgE antibody, delivered by the aerosol route was no better than aerosolized placebo in attenuating the airway responses to inhaled allergen in allergic asthmatic subjects. This result is in contrast to previous findings in protocols where E25 was delivered intravenously.

The early phase response to allergen at Weeks 2 and 8 after initiation of treatment with aerosolized E25-1 mg daily was significantly less than the baseline early phase response, but the degree of attenuation was not significantly greater than placebo. The effects of E25-10 mg on the early phase response were no greater than the effects of E25-1 mg and also no greater than placebo. Similarly, analysis of data for the late phase response in the subgroup of subjects who had a significant late phase response at baseline showed no significant attenuation for either the 1 mg or the 10 mg dose groups. These data for the late phase response have to be interpreted very cautiously because of the small number of subjects with a late phrase response at baseline. Previously, in similar protocols using similar methods, we have shown that E25 administered intravenously significantly reduces both the early and late phase responses to inhaled allergen (1, 2). Thus, the aerosol route of delivery for E25 is not as effective as the intravenous route in attenuating airway responses to inhaled allergen.

There are at least three reasons for the lack of efficacy of aerosolized E25 in this study. First, the aerosol route of delivery may not have delivered sufficient E25 to the lower airways. E25 clearly reached the lower airways after aerosolization because E25 was detected in BAL and blood, and any swallowed E25 would have been inactivated in the stomach. However, it is possible that the aerosol route of delivery did not result in high enough concentrations of E25 to neutralize IgE in the lung tissue compartments surrounding IgE effector cells. The vascular space, in particular, represents a large "sink" of IgE constantly available to move into the lung interstitium to replace IgE complexed with E25. A second possible explanation for the lack of efficacy of aerosolized E25 in this study is that neutralizing antibodies to E25 developed and prevented E25 from binding free IgE. Antihuman antibodies have never been detected after intravenous administration of humanized E25 that contains less than 5% murine amino acid. However, the aerosol route of administration of E25 may be more immunogenic than the intravenous or subcutaneous route. A third possible explanation for the lack of efficacy of aerosolized E25 in this study is that the subjects were noncompliant. Compliance appears to have been good in this study, however, as evidenced by the counts of used and unused medication vials at each study visit.

In summary, we found that aerosolized E25 did not significantly attenuate the airway responses to inhaled allergen in asthmatic subjects. One subject developed an IgG and IgA antibody against E25, suggesting that the aerosol route of delivery for monoclonal antibodies may be more immunogenic than the intravenous or subcutaneous route. We conclude that the aerosol route of delivery for E25 is not likely to be a useful treatment for allergic asthma.

***Acknowledgment***: The writers are indebted to Paula Jardieu, Ph.D., Genentech Inc., and Theresa Sweeney, Genentech Inc., for directing the preclinical phase of development of E25. In addition, they are grateful to Robert Fick, M.D., Genentech Inc., and Homer Boushey, M.D., UCSF, for their advice and assistance with protocol development and to Michel Laviolette, M.D., for performing the bronchoscopy procedures.

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US006165463A

# United States Patent [19]

**Platz et al.**

[11] **Patent Number: 6,165,463**

[45] **Date of Patent: Dec. 26, 2000**

[54] **DISPERSIBLE ANTIBODY COMPOSITIONS AND METHODS FOR THEIR PREPARATION AND USE**

[75] Inventors: **Robert M. Platz**, Half Moon Bay; **John S. Patton**, San Carlos; **Linda C. Foster**, Mountain View, all of Calif.; **Mohammed Eljamal**, Tripoli, Lebanon

[73] Assignee: **Inhale Therapeutic Systems, Inc.**, San Carlos, Calif.

[21] Appl. No.: **09/323,276**

[22] Filed: **Jun. 1, 1999**

**Related U.S. Application Data**

[62] Continuation of application No. 08/951,312, Oct. 16, 1997, which is a continuation-in-part of application No. 08/423,515, filed as application No. PCT/US96/05070, Apr. 12, 1996.

[51] **Int. Cl.**[7] ..... **A61K 39/395**

[52] **U.S. Cl.** ..... **424/130.1**; 514/951

[58] **Field of Search** ..... 514/535, 171, 514/951; 424/130.1

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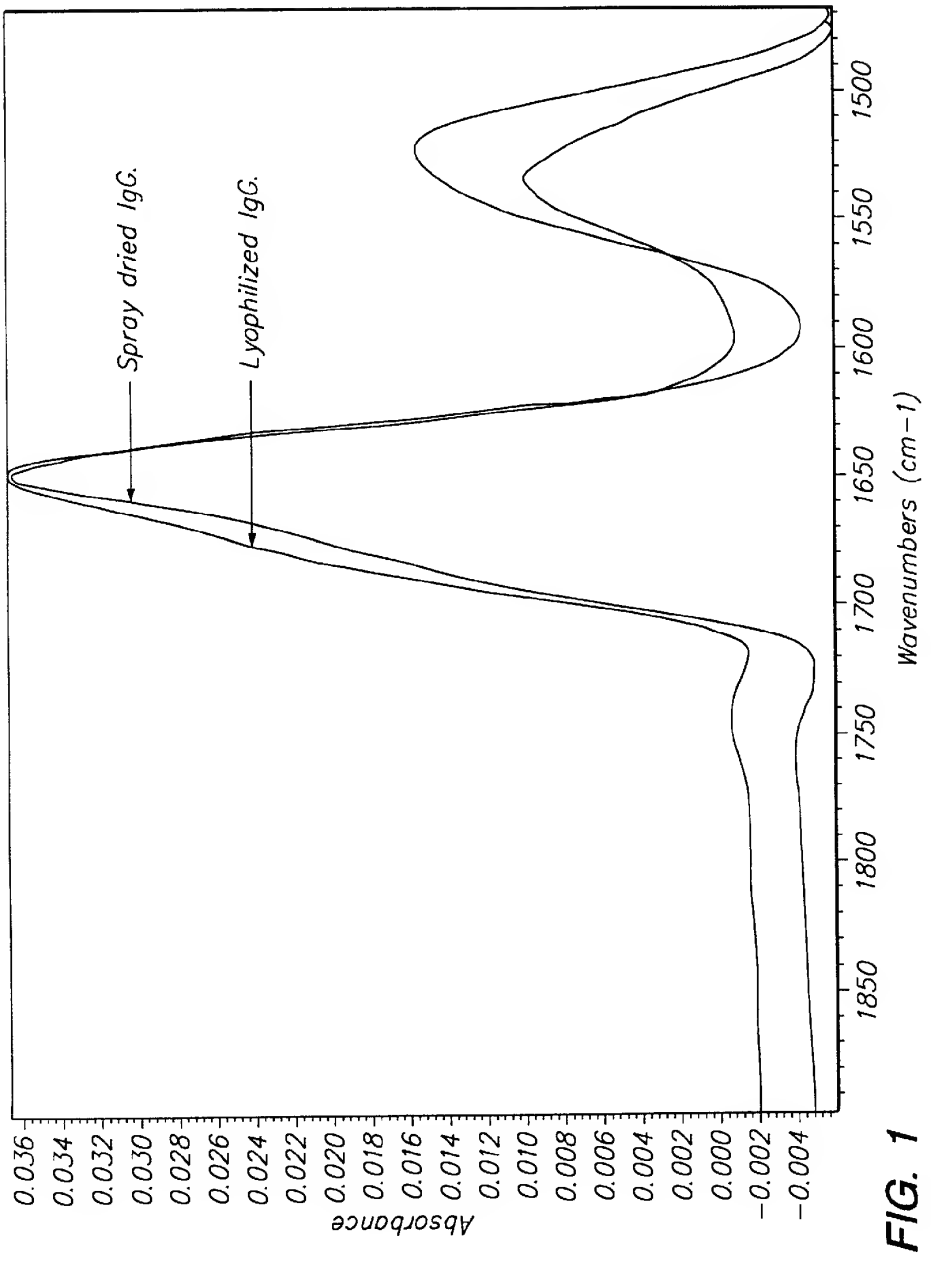
*Primary Examiner*—Jose' G. Dees
*Assistant Examiner*—Alton Pryor
*Attorney, Agent, or Firm*—Burns, Doane, Swecker & Mathis, L.L.P.

[57] **ABSTRACT**

This invention relates to dispersible antibody compositions and methods for preparing and using these compositions. In particular, the present invention relates to dry powder dispersible antibody compositions wherein antibody conformation is preserved. The compositions have good powder dispersibility and other desirable characteristics for pulmonary delivery of therapeutic antibodies.

**14 Claims, 4 Drawing Sheets**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Lyophilized IgG.
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0.030
0.028
0.026
0.024
0.022
0.020
0.018
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
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1750
1700
1650
1600
1550
1500
Wavenumbers (cm−1)

FIG. 1

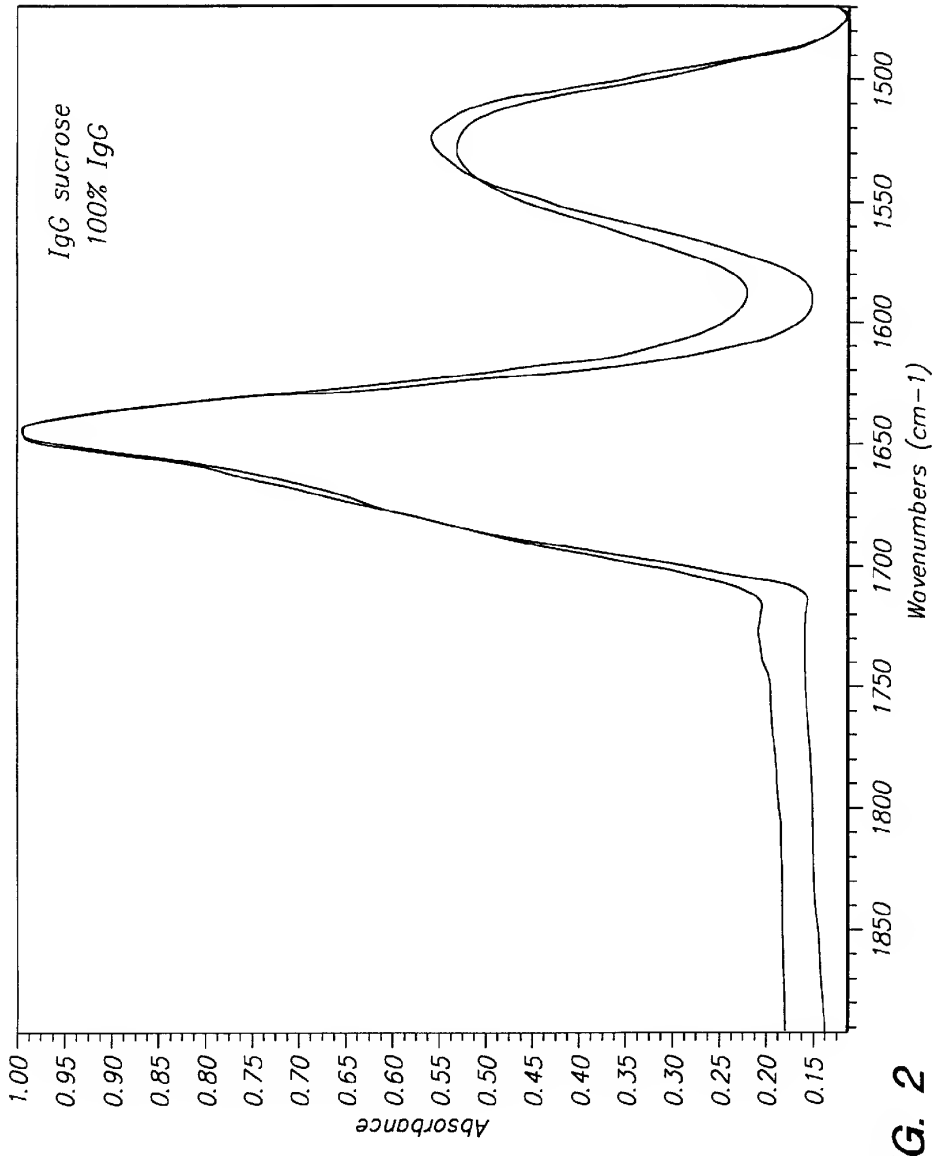
IgG sucrose
100% IgG
Absorbance
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
1850
1800
1750
1700
1650
1600
1550
1500
Wavenumbers (cm-1)

FIG. 2

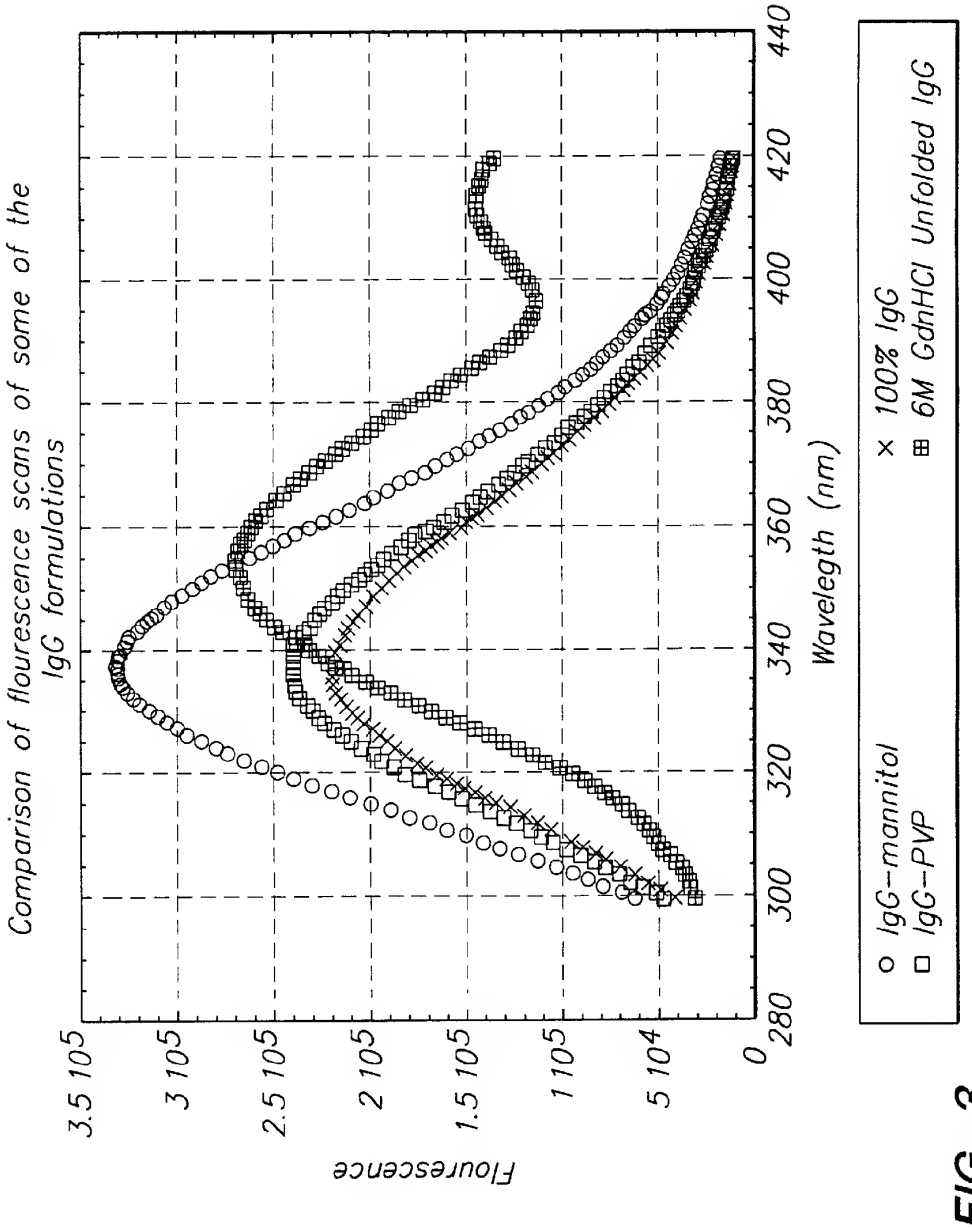
Comparison of flourescence scans of some of the IgG formulations
Fluorescence
3.5 10^5
3 10^5
2.5 10^5
2 10^5
1.5 10^5
1 10^5
5 10^4
0
280
300
320
340
360
380
400
420
440
Wavelegth (nm)
o IgG–mannitol
□ IgG–PVP
× 100% IgG
⊞ 6M GdnHCl Unfolded IgG

FIG. 3

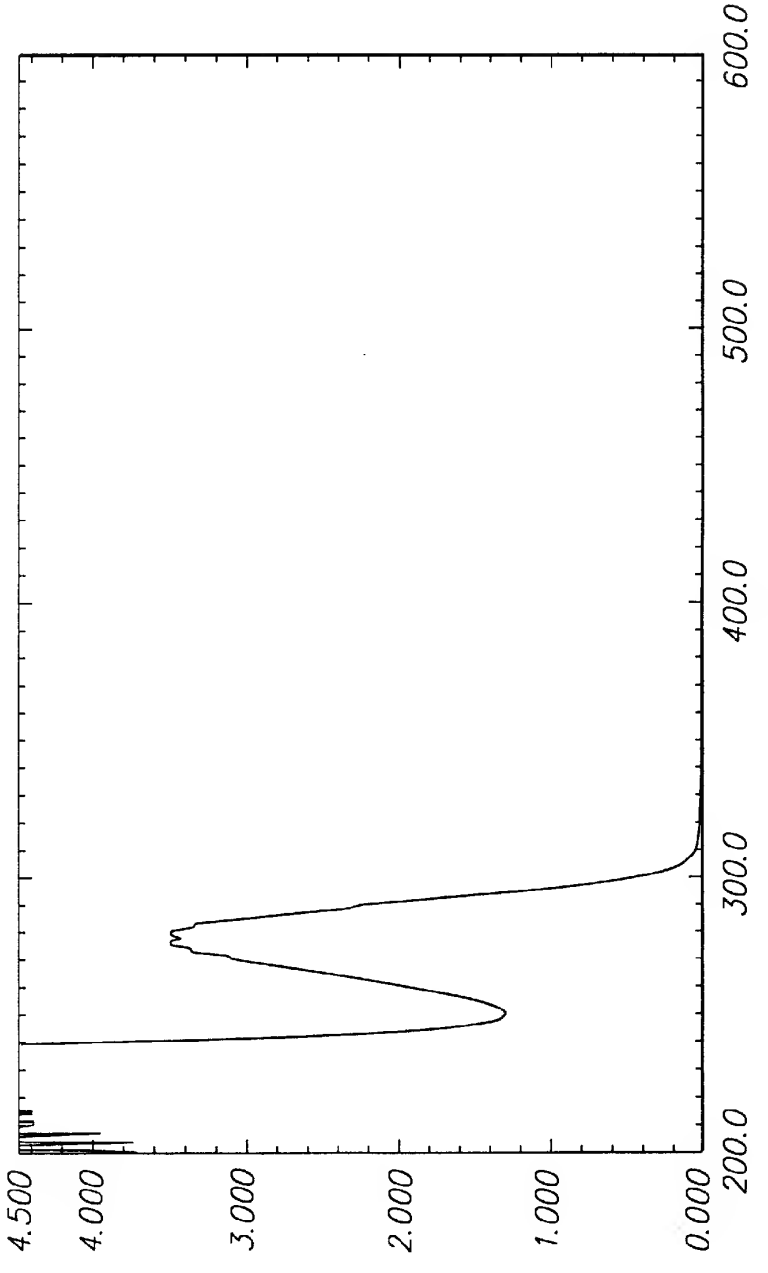
4.500
4.000
3.000
2.000
1.000
0.000
200.0
300.0
400.0
500.0
600.0
Wavelegth (nm)

FIG. 4

# DISPERSIBLE ANTIBODY COMPOSITIONS AND METHODS FOR THEIR PREPARATION AND USE

## CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 08/951,312 filed Oct. 16, 1997. This application is a continuation-in-part of U.S. application Ser. No. 08/423,515 filed Apr. 14, 1995 and PCT Application No. PCT/US96/05070 filed Apr. 12, 1996, which applications are incorporated by reference herein in their entirety.

## FIELD OF THE INVENTION

This invention relates to dispersible antibody compositions and methods for preparing and using these compositions. In particular, the present invention relates to dry powder dispersible antibody compositions wherein antibody conformation is preserved. The compositions have good powder dispersibility and other desirable characteristics for pulmonary delivery of therapeutic antibodies.

## BACKGROUND OF THE INVENTION

The protective effects of humoral immunity are known to be mediated by a family of structurally related glycoproteins called antibodies. Antibodies initiate their biological activity by binding to antigens. Antibody binding to antigens, which may be covalent or noncovalent, is exquisitely specific for one antigen and is often very strong.

Antibodies are produced in a membrane-bound form by B-lymphocytes. Blood contains many different antibodies, each derived from a clone of B-cells and each having a distinct structure and specificity for antigen. Antibodies are present in the cytoplasmic compartment and on the surface of B-lymphocytes, in the plasma, in interstitial fluid of the tissues and in secretory fluids such as saliva and mucous. Surfaces of immunoeffector cells, such as mononuclear phagocytes, natural killer cells and mast cells also have antibodies.

All antibodies are similar in their overall structure, accounting for certain similarities in physiochemical features such as charge and solubility. All antibodies have a common core structure of two identical light chains, each about 24 kilodaltons, and two identical heavy chains of about 55–70 kilodaltons each. One light chain is attached to each heavy chain, and the two heavy chains are attached to each other. Both the light and heavy chains contain a series of repeating homologous units, each of about 110 amino acid residues in length which fold independently in a common globular motif, called an immunoglobulin (Ig) domain. The region of an antibody formed by the association of the two heavy chains is hydrophobic. Antibodies, and especially monoclonal antibodies, are known to cleave at the site where the light chain attaches to the heavy chain when they are subjected to adverse physical or chemical conditions. Because antibodies contain numerous cysteine residues, they have many cysteine-cysteine disulfide bonds. All Ig domains contain two layers of beta-pleated sheets with three or four strands of anti-parallel polypeptide chains.

Despite their overall similarity, antibody molecules can be divided into a small number of distinct classes and subclasses based on physiochemical characteristics such as size, charge and solubility, and on their behavior in binding to antigens. In humans, the classes of antibody molecules are:



IgA, IgD, IgE, IgG and IgM. Members of each class are said to be of the same isotype. IgA and IgG isotypes are further subdivided into subtypes called $IgA_1$, $IgA_2$ and $IgG_1$, $IgG_2$, $IgG_3$ and $IgG_4$. The heavy chains of all antibodies in an isotype share extensive regions of amino acid sequence identity, but differ from antibodies belonging to other isotypes or subtypes. Heavy chains are designated by the letters of the greek alphabet corresponding to the overall isotype of the antibody, e.g., IgA contains $\alpha$, IgD contains $\delta$, IgE contains $\epsilon$, IgG contains $\gamma$, and IgM contains $\mu$ heavy chains. IgG, IgE and IgD circulate as monomers, whereas secreted forms of IgA and IgM are dimers and pentamers, respectively, stabilized by the J chain. Some IgA molecules exist as trimers.

There are $1\times10^7$, and perhaps as many at $10^9$, structurally different antibody molecules in every individual, each with the unique amino acid sequence in their antigen combining sites. Sequence diversity in antibodies is confined to three short stretches within the amino terminal domains of the heavy and light chains. The amino acid sequences of the amino terminal domains are called variable (V) regions, to distinguish them from the more conserved constant (C) regions.

Antibodies have several known therapeutic applications. For example, they may be used to bind to and block cell markers and receptors. Antibodies to microorganisms may be used to inhibit or inactivate the microorganism and/or prevent or treat disease conditions caused by these microorganisms. When antibodies bind to microorganisms, they enhance their recognition and destruction by macrophages. Antibodies may be used as anticytokines, antichemokines, antihormones, antiinflammatories and immunosuppressors or as antineutrophil adhesion agents. Antibodies, especially monoclonal antibodies, may be used systemically to deliver therapy. In these cases antibodies are often used as immunoconjugates, immunoliposomes or immunomicrospheres. Antibodies can be used as apoptosis stimulators and as recognizers of cancerous and precancerous cells. Known examples of antibodies which may be useful therapeutically include the following.

MedImmune Inc. is studying the use of humanized anti-RSV monoclonal antibodies and markets a polyclonal anti-RSV antibody from donor blood (RespiGam) to treat respiratory syncytial virus (RSV) infections. MedImmune also markets CytoGam, an anti-CMV (cytomegalovirus) human immune globulin for the treatment of CMV infection. IDEC and Genentech are jointly performing clinical trials of a chimeric mouse-human monoclonal antibody (rituximab) aimed at the CD20 antigen found on mature B cells and most non-Hodgkin's lymphoma tumors for use in treating relapsed or refractory low-grade non-Hodgkin's lymphoma. GalaGen is studying the use of the polyclonal antibody Diffistat-G for treatment of Clostridium difficile antibiotic associated diarrhea. Smith Kline and Schering Plough are developing an anti-IL-5 antibody which has been shown in clinical trials to prevent eosinophilic inflammation and airway constriction. An anti-IgE antibody is being developed by Genentech to "switch-off" allergies. Monoclonal antibody Rhu-Mab-E25, which is a humanized chimeric $IgG_1$ monoclonal antibody for a unique epitope on human high affinity IgE receptors (FceRI), has been shown to reduce free IgE levels after the first administration by injection. It attenuated both early and late phase responses to inhaled allergens after multiple injections. Examples of antibodies used therapeutically also include a nebulized IgG (Sandoz), which is used intranasally against respiratory syncytial virus (RSV); IINK20 (Oravax), an anti-RSV IgA; and 4B9

(Bristol Myers-Squibb), an anti-group B Streptococcus IgM monoclonal antibody. Other therapeutically useful antibodies include anti-CD4 antibodies, anti-IL-2 antibodies and anti-IL4 antibodies.

The immunotherapy of respiratory syncytial virus infection using small particle aerosols of IgG has been disclosed by Piazza et al. (The Journal of Infectious Diseases, Vol. 166, pp. 1422–1424, 1992) In this study it was shown that a 15-minute exposure to an aerosolized 5% solution of IgG effected a 50-fold reduction in pulmonary virus. Brown (Aerosol Science and Technology, Vol. 24, pp. 45–56, 1996) discloses the use of antibodies as inhibitors or antagonists of cytokines to depress respiratory inflammatory diseases or allergen-induced asthmatic responses. Also disclosed is local respiratory delivery of pathogen-specific antibody for treatment of acute viral or bacterial respiratory infections. Antibody liposomes, i.e., immunoliposomes, are disclosed by Maruyama et al. in Biochemica et Biophysica Acta, Vol. 1234, pp. 74–80, 1995. Coating liposomes with antibody leads to enhanced uptake of the immunoliposome by the reticuloendothelial system. Human monoclonal antibodies are known to be useful as antitumor agents. A mouse/human monoclonal $IgG_1$ antibody specific for the Lewis Y antigen found on the surface of tumor cells is disclosed by Paborji et al. (Pharmaceutical Research, Vol. 11, No. 5, pp. 764–771, 1994). The use of antibodies in metered-dose propellant driven aerosols for passive antibody aerosol therapy against respiratory infections is disclosed in Brown et al. (Journal of Immunological Methods, Vol. 176, pp. 203–212, 1994). Immune responses in the respiratory tract are of great importance for protection against infections of the respiratory system and for their involvement in respiratory allergies and asthma. Effective targeting of immunomodulating reagents including antibodies to the respiratory tract is shown to be of benefit in increasing local immunity to respiratory pathogens or decreasing immune mediated respiratory pathology. Inhaled immunoconjugates, immunoliposomes or immunomicrospheres have application in the lung as killers of cancer cells (immunoconjugates) or, in the case of immunoliposomes and microspheres, as stealth delivery particles of a variety of therapeutic agents. An IgM anti-group B Streptococcus monoclonal antibody is disclosed by Gombotz et al. (Pharmaceutical Research, Vol. 11, pp. 624–632, 1994).

Over the years certain drugs have been sold in compositions suitable for forming a drug dispersion for oral inhalation (pulmonary delivery) to treat various conditions in humans. Such pulmonary drug delivery compositions are designed to be delivered by inhalation by the patient of the drug dispersion so that the active drug within the dispersion can reach the lung. It has been found that certain drugs delivered to the lung are readily absorbed by the alveolar region directly into the blood circulation. Pulmonary delivery is particular promising for delivery of macromolecules such as proteins, polypeptides, high molecular weight polysaccharides and nucleic acids, which are difficult to deliver by other routes of administration. Such pulmonary delivery can be effective both for systemic delivery and for localized delivery to treat diseases of the lungs.

Pulmonary drug delivery can itself be achieved by different approaches, including liquid nebulizers, aerosol-based metered-dose inhalers (MDI's) and dry powdered dispersion devices. Chlorofluorocarbon (CFC) based MDI's are losing favor because of their adverse effect on the environment. Dry powder dispersion devices, which do not rely on CFC aerosol technology, are promising for delivering drugs that may be readily formulated as dry powders. Many otherwise labile macromolecules may be stably stored as lyophilized or spray dried powders, either by themselves or in combination with suitable powder carriers.

The ability to deliver pharmaceutical compositions as dry powders, however, is problematic in certain respects. The dosage of many pharmaceutical compositions is often critical, so it is desirable that dry powder delivery system be able to accurately, precisely and reliably deliver the intended amount of drug. Moreover, many pharmaceutical compositions, including antibodies, are quite expensive. Thus, the ability to efficiently formulate, process, package and deliver the dry powders with minimal loss of drug is critical. While the permeability of natural macromolecules in the lung is well known, combined inefficiency of macromolecule production processes and macromolecule delivery has limited commercialization of dry macromolecule powders for pulmonary delivery. It is also essential that dry powders for pulmonary delivery be readily dispersible prior to inhalation by the patient in order to assure adequate distribution and systemic absorption.

An important requirement for hand held and other powder delivery devices is efficiency. It is important that the delivered dose be relatively high to reduce the number of breaths required to achieve a total dosage. The ability to achieve both adequate dispersion and small dispersed volumes is a significant technical challenge that requires in part that each unit dosage of the powder composition be readily and reliably dispersible. Certain pulmonary delivery devices, such as those disclosed in U.S. Pat. No. 5,458,135 and International Patent Publication WO96/09085 are useful for pulmonary delivery of dry powder drugs.

Spray drying is a conventional chemical processing operation used to produce dry particulate solids from a variety of liquid and slurry starting materials. The use of spray drying for the formulation of dry powder pharmaceuticals is known but has usually been limited to small molecule and other stable drugs which are less sensitive to thermal degradation and other rigorous treatment conditions. The use of spray drying for the preparation of biological macromolecule compositions, including antibodies, can be problematic since such macromolecules are often labile and subject to degradation when exposed to high temperatures and other aspects of the spray drying process. Excessive degradation of the macromolecules can lead to drug formulations lacking in the requisite purity.

It can also be difficult to control particle size and particle size distribution in compositions produced by spray drying. For pulmonary delivery it is critical that the average particle size be maintained in a respirable range and that the amount of the composition comprising particles outside the target size range be minimized. Moreover, it can sometimes be difficult to achieve a desired low moisture content required for physical and chemical stability in the final particulate product, particularly in an economic manner. Finally, and perhaps most importantly, it has been difficult to produce the small particles necessary for pulmonary delivery in an efficient manner. For high value macromolecular drugs, high collection efficiencies, i.e., the amount of particulate drug recovered from the process in a usable form, are important. While spray drying has been used to prepare powders of macromolecules in laboratory scale equipment, commercial spray dryers are not designed to produce powders in the pulmonary size range. The methods for atomization, drying powder and collection must be modified to economically produce a protein powder with the desired product characteristics for pulmonary delivery and in sufficient yield and at commercially acceptable production rates, i.e., in excess of

30 grams per hour. Useful methods are disclosed, for example, in International Patent Application No. PCT/US97/07779, the disclosure of which is incorporated herein by reference in its entirety.

## SUMMARY OF THE INVENTION

The present invention provides dispersible dry powder antibody compositions and methods for their preparation and use. These dispersible dry powder antibody compositions have a moisture content of less than about 10% by weight (%w) water, usually about 1–5% by weight, and preferably less that about 3% by weight of water. They have a particle size of about 0.1 to 7 μm mass median diameter (MMD), usually about 0.4 to 5 μm MMD, preferably about 1 to 4 μm and most preferably about 1–3 μm MMD. The dispersible dry powder antibody compositions of the present invention result in a delivered dose of greater than about 30%, usually greater than about 40%, preferably greater than about 50% and most preferably greater than about 60%. They have an aerosol particle size distribution of about 1–5 μm mass median aerodynamic diameter (MMAD), usually about 1.5–4.5 μm MMAD and preferably about 1.5–4.0 μm MMAD, or with at least about 40% (preferably at least about 50%) of the particles less than about 3.3 μm in diameter. The compositions contain at least about 40% by weight antibody. Further, the antibodies in these compositions are not aggregated and are present in their native conformation so as to retain their biological activity.

According to the present invention, antibody containing compositions having improved characteristics which overcome at least some of the deficiencies noted above with respect to prior compositions are provided. The present invention provides compositions and methods which provide a predetermined concentration of antibody, and, optionally, other excipients as a dry dispersible powder. The antibody is optionally formulated prior to spray drying with compatible excipients such as sugars, buffers, salts, surfactants, polymers, other proteins and other specific stabilizing additives as needed to provide a therapeutically effective dose, inhibit degradation during drying, promote powder dispersibility and achieve acceptable physical and chemical stability of the powder at room temperature while maintaining antibody integrity.

In one aspect the invention provides dry powder compositions for pulmonary delivery comprising an antibody that is substantially in its native conformation. Such compositions retain antibody activity upon reconstitution.

In another aspect the invention provides antibody-based dry powder compositions which are spray dried from the antibody and, optionally, excipient, in a solvent under conditions to provide a respirable dry powder. Solvents for such mixtures may include water and ethanol.

In a yet further aspect, the invention provides methods for preventing or treating a condition by administering the dry powder antibody compositions described above.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** illustrates comparative FT-IR spectra of spray dried and lyophilized 100% IgG. This figure shows that the spray dried powder retained antibody integrity.

FIG. **2** illustrates comparative FT-IR spectra of 100% IgG and IgG:sucrose:citrate (70:20:10) powders, both of which retained native conformation.

FIG. **3** is a fluorescence scan of some antibody powders of the present invention. No alteration in antibody conformation after spray drying was seen.

FIG. **4** illustrates a UV spectrum of an IgG:mannitol:citrate (70:20:10) powder after reconstitution. No light scattering at 400 nm was seen, indicating the absence of aggregation.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention is based at least in part on the discovery that antibodies may be formulated as dispersible dry powder compositions while retaining their integrity. The dispersibility characteristics of the subject antibody based compositions means that they are more suitable for use in pulmonary delivery devices than antibody compositions prepared by other methods. The compositions of the invention are readily aerosolized and rapidly absorbed through the lungs of a host when delivered by a dry powder inhaler. Standard lyophilized antibody formulations do not consist of particles with size suitable for pulmonary delivery. In contrast, the dry powder formulations of the present invention retain antibody conformation and stability, are readily dispersible for pulmonary delivery and allow for unit dose packaging.

The invention consists of compositions comprising antibodies in dry powder dispersible formulations. The use of dry particles of a certain size range allows for delivery of antibodies to the alveolar area of the lungs. Optionally, the dry powder formulations of the present invention may contain stabilizers and excipients such as sugars, polymers and other proteins. The compositions of the present invention are useful in pulmonary dry powder drug delivery systems, including but not limited to those disclosed in U.S. Pat. No. 5,458,135 and International Patent Publication WO96/09085.

A. Definitions

As used herein the following terms have the following meanings:

The terms "dispersibility" or "dispersible" mean a dry powder having a moisture content of less than about 10% by weight (%w) water, usually below about 5%w and preferably less that about 3%w; a particle size of about 0.4 to 5 μm mass median diameter (MMD), usually about 1 to 4 μm MMD and preferably 1–3 μm MMD; a delivered dose of greater than about 30%, usually greater than about 40%, preferably greater than about 50% and most preferably greater than about 60%; and an aerosol particle size distribution of about 1–5 μm mass median aerodynamic diameter (MMAD), usually about 1.5–4.5 μm MMAD and preferable about 1.5–4.0 μm MMAD, or with at least about 40% (preferably at least about 50%) of the particles less than about 3.3 μm in diameter.

The term "non-aggregated" means that the antibody is substantially oligomer-free. This means that usually less than about 15%, preferably less than about 10% and more preferably less than about 5% of the antibody in the composition comprises covalently or non-covalently bound dimers, trimers or larger aggregates.

The term "native conformation" means that the antibody in the composition substantially retains the secondary and tertiary structure of the original state of the molecule.

The term "powder" means a composition that consists of finely dispersed solid particles that are free flowing and capable of being readily dispersed in an inhalation device and subsequently inhaled by a subject so that the particles reach the spaces of the deep lung to permit penetration into the alveoli. Thus, the powder is said to be "respirable."

The term "dry" means that the composition has a moisture content such that the particles are readily dispersible in an

inhalation device to form an aerosol in the absence of a liquid propellant. This moisture content is generally below about 10%w water, usually below about 5%w and preferably less than about 3%w.

The terms "pharmaceutical excipient" or "additive" mean compounds which stabilize antibody and/or improve powder aerosol performance and stability. The types of excipients useful in the present invention include proteins such as human serum albumin (HSA), carbohydrates such as sucrose, alditols such as mannitol, salts such as sodium citrate, polymers such as PVP and Ficoll, and the like.

The term "antibody" means the structurally related glycoproteins which bind to antigens. The term antibody includes monoclonal antibodies and/or IgA, IgD, IgE, IgG and IgM isotype antibodies, including these compounds present in the form of immunoconjugates, immunoliposomes or immunospheres.

B. Compositions:

The present invention is drawn to dispersible antibody-containing dry powder compositions suitable for pulmonary delivery. The compositions comprise a therapeutically effective amount of an antibody, optionally in combination with a pharmaceutically acceptable carrier or excipient.

These dry powder compositions comprise a plurality of discrete, dry particles with an average particle size below about 7 $\mu$m, preferably in the range from 0.4–5 $\mu$m, more preferably from 1–4 $\mu$m and most preferably from 1–$\mu$m. The average particle size of the powder is measured as mass mean diameter (MMD). Such powders are capable of being readily dispersed in an inhalation device and subsequently inhaled by a patient so that the particles are able to reach the alveolar regions of the lungs.

A particular characteristic which relates directly to improved dispersibility and handling characteristics of respirable dry powders is rugosity. Rugosity is the ratio of the specific area (measured by molecular surface adsorption or another known technique) and the surface area calculated from particle size distribution (as measured by a centrifugal sedimentary particle size analyzer) and particle density (as measured by pycnometry), assuming non-porous spherical particles. If the particles are known to be generally nodular in shape, as spray dried particles are, rugosity is a measure of the degree of convolution or folding of the surface. A rugosity of 1 indicates that the particle is spherical and non-porous. Rugosity values greater than 1 indicate that the particle surface is non-uniform and convoluted to at least some extent, with higher numbers indicating a higher degree of non-uniformity. For the antibody-based powders of the present invention, it has been found that particles preferably have a rugosity of at least 2, more preferably of at least 3.

Prior dispersible dry powder formulations of macromolecules have shown that certain peptides and proteins could be formulated into dispersible dry powder compositions suitable for pulmonary delivery. However, the molecular weight of the peptides and proteins previously formulated is lower than the molecular weight of the antibodies formulated in the present invention, which have molecular weights of from about 150 to about 400 kilodaltons. It is well known that larger proteins are subject to degradation such as cleavage due to physical stress. Thus, they are not easily formulated into dispersible dry powders suitable for pulmonary delivery. Accordingly, it is unexpected that antibodies can be so formulated, as shown in the present invention.

The moisture content of the dry powder particles of the present invention is usually below about 10% by weight water, preferably below about 5%w and more preferably below about 3%w. Such low moisture content powders are generally physically and chemically stable during storage at room temperature and are readily dispersible in an inhalation device to form an aerosol.

Examples of antibodies which may be formulated using the present invention include those which have biological activity or which may be used to treat a disease or other condition. They include, but are not limited to, antibodies to microorganisms (including respiratory pathogens), monoclonal antibodies directed against tumor antigens and antibodies to cell receptors (including receptors involved in inflammation and allergy). Immunoconjugates of each of these examples may also be formulated using the present invention. Analogs, derivatives, fragments and pharmaceutically acceptable salts of the above may also be used. They may also be formulated with lipids, liposomes, microspheres or the like.

Antibodies suitable for use in the compositions of this invention include IgA, IgE, IgG, IgD and IgM. IgA, IgG and IgM antibodies are preferred, with IgG and IgA antibodies being particularly preferred. The amount of antibody which constitutes a therapeutically effective amount will vary in the composition depending on the biological activity of the antibody employed and the amount needed in the unit dosage form. The condition to be treated or prevented will also determine the amount of antibody required, as will the subject to which the antibody composition is being administered. Because antibodies are generally low potency drugs, the compositions comprise at least about 40% by weight antibody in the formulation, preferably between about 70% to about 100% and most preferably about 70% to about 90%. The amount of excipients and pharmaceutically acceptable carriers may be from about 0–60%, preferably from about 0–30% and most preferably from about 10–30% by weight.

Compositions according to the present invention comprise dispersible antibody powders intended for pulmonary delivery, i.e., inhalation by a patient into the alveolar regions of the patients lungs. The compositions comprise particles having an average particle size below about 10 $\mu$m. Preferably the particles of the composition will have a moisture content below about 10% by weight, more preferably below about 5% by weight and typically below about 3% by weight. Preferably at least about 50% by weight of the composition will comprise particles having a particle size less than about 5 $\mu$m, more preferably 75% of particles in the range from 0.4 $\mu$m to 5 $\mu$m. The compositions will often be packaged as unit doses where a therapeutically effective amount of the antibody composition is present in a unit dose receptacle, such as a blister pack, gelatin capsule, or the like, so long as a moisture barrier is provided.

Pharmaceutical excipients and/or additives useful in the present invention include proteins (e.g., HSA, recombinant human albumin (rHA), gelatin and casein), peptides (e.g., aspartame) and amino acids (e.g., alanine, glycine, arginine, glutamic acid and aspartic acid) which improve conformational stability of antibody during spray drying and also improve dispersibility of the powder. Carbohydrates/sugars and alditols are also particularly useful. Suitable carbohydrate/sugar compounds include sucrose, trehalose, lactose, raffinose, and the like. Suitable alditols include mannitol and pyranosyl sorbitol and the like. Polymeric excipients/additives include polyvinylpyrrolidones (PVP), Ficolls, soluble hydroxy ethyl starch, dextrates and the like of suitable molecular weight. Suitable pH adjusters or buffers include organic salts prepared from organic acids and bases, such as sodium citrate, glycine, sodium tartrate, sodium lactate, tromethamine and the like. Also useful are

small amounts of pharmaceutically acceptable surfactants such as Tweens, chelators such as EDTA and inorganic acids and bases such as sodium phosphate and the like. Other suitable pharmaceutical excipients and/or additives include those disclosed in *Pharmaceutical Sciences*, Remington, 18th ed. (1990), the disclosure of which is incorporated herein by reference.

The antibody-based dry powder compositions of the present invention may be produced by spray drying solutions or slurries of the antibody and, optionally, excipients, in a solvent under conditions to provide a respirable dry powder. Solvents may include polar compounds such as water and ethanol, which may be readily dried. Antibody stability may be enhanced by performing the spray drying procedures in the absence of oxygen, such as under a nitrogen blanket or by using nitrogen as the drying gas.

Spray drying IgG with the optimized conditions described in the Examples section produced antibody-containing powders with particle size distribution between 1.2–1.8 μm MMD. The moisture content of these powders ranged from 1.3–3.5% by weight.

## DISCLOSURE OF THE EXAMPLES OF THE INVENTION

The following examples are not intended to limit the scope of the invention in any manner.

Materials and Methods:

In general the following materials and methods were used in the examples that follow unless otherwise indicated.

Materials:

IgG, a glyocosylated protein of 150 kilodalton molecular weight purified from pooled normal human serum by fractionation on ion-exchange chromatography was purchased from Sigma. The IgG used was an essentially salt-free (less than 1% sodium), lyophilized bulk substance.

Formulation excipients were research grade or better. Citrate buffer salts used in formulation preparation were USP/ACS grade. The following excipients were used in preparing the IgG powders: Recombinant human serum albumin (Miles); Citric acid, monohydrate (JT Baker); Trisodium citrate, dihydrate (YT Baker); sucrose (EM Science); mannitol (USP); polyvinylpyrrolidone (PVP) k-15, molecular weight 10 kilodaltons (ISP Tech) and Ficoll (Pharmacia), a non-ionic synthetic polymer of sucrose, molecular weight 400 kilodaltons.

Analytical reagents used were reagent grade or better. Research grade dimethylformamide was used for moisture content analysis.

Sample Preparation and Handling:

Spray-dried powders were stored and prepared for analytical testing in a glove box maintained with dry air atmosphere. During powder transfer, the dry box was maintained at less than 5% relative humidity.

Physical Methods:

Moisture content

The water content of the powder formulations was determined by coulometric Karl Fischer titration using a Mitsubishi Model CA-06 moisture meter. A 5–10 mg aliquot of powder was dissolved in 1 ml of dimethylformamide (DMF). The sample was then injected into the titration cell of the moisture meter.

Powder particle size distribution

The particle size distribution (PSD) of the spray dried powder samples was measured with a Horiba CAPA-700 centrifugal sedimentation particle size analyzer. Approximately 2–3 mg of powder was suspended in 2–3 ml of Sedisperse A-11 (Micromeritics, Norcross, Ga.) and sonicated briefly (5 minutes) before analysis. The instrument was configured to measure a particle size range of 0.4 to 10 μm in diameter (MMD). A particle density of 1.44 g/cm$^3$ was used for analyzing these powders.

Aerosol Methods:

Delivered dose assay

Delivered dose assay was performed to determine the efficiency and reproducibility of pulmonary delivery of the dispersible dry powder antibody composition. The device used was similar to devices disclosed in WO96/09085. The delivered dose was measured by collecting the aerosol on a filter placed over the mouthpiece of the chamber of the device. The powder sample was collected on a glass fiber filter (Gelman, 47 mm diameter) by drawing the aerosol from the chamber at an airflow of 30 L/minute for 2.5 seconds. Delivered dose efficiency was calculated by dividing the mass of the powder collected on the filter by the mass of the powder in the blister pack. Each result was the average of five replicate measurements.

Aerosol particle size distribution

The aerosol particle size distribution was obtained using an eight stage cascade impactor (Graseby Andersen, Smyrna, Ga.). The impactor air-flow was set to 28.3 L/min., the calibrated flow-rate for the instrument. For each run, 5 blister packs filled with approximately 5 mg of powder was dispersed from the inhaler. The particle size was determined by weighing the powder on the impactor plates and evaluating the results on a log-probability plot. Both the mass median aerodynamic diameter (MMAD) and the mass fraction less than 5 μm were determined from the log-probability plot.

The mass fraction of the aerosol powder less than 3.3 μm was obtained using a 2-stage (3.3 μm and 0.4 μm cut size) cascade impactor (short-stack Andersen). The impactor air-flow was set to 28.3 L/minute, the calibrated operating flow rate of the instrument. For each measurement, one blister pack filled with a known weight of powder was dispersed from the inhaler. The resultant aerosol was drawn from the device chamber into the cascade impactor. The particle size fractions were determined by weighing the powder on the impactor filters. The % of mass <3.3 μm was determined based upon the weights of those filters.

Biophysical methods:

Attenuated Total Reflectance Fourier Transform—IR (ATR FT-IR)

Infrared (IR) spectroscopy can provide information regarding the secondary structure of proteins. The majority of the protein structural information, however, has been obtained from one absorbance originating primarily from the amide C=O stretching vibration: the amide I band. The sensitivity to variations in both geometric arrangements of atoms and hydrogen bonding enables infrared spectroscopy to discriminate between the various secondary structures incorporated within the three-dimensional organization of peptides and proteins, e.g., helical, extended sheet, disordered and turns. Solid samples, e.g., powders, which are not transparent are analyzed using ATR-IR. The basis for ATR-IR is that the IR beam enters an optically transparent medium, in this case Germanium crystal, on which the sample is layered. Comparison FT-IR spectra of spray dried and lyophilized powders were analyzed for differences which would indicate conformational changes due to spray drying. FIG. **1** shows the FT-IR spectra of lyophilized IgG and spray dried IgG. No significant difference in the spectra shape or peak maxima were noted, indicating that the spray dried IgG powder retained its conformational integrity. FIG. **2** shows that both a 100% IgG powder formulation and an

IgG:sucrose:citrate (70:20:10) powder retained native conformation of antibody.

Intrinsic Tryptophan Fluorescence

For most proteins, aromatic amino acid residues like tryptophan, tyrosine and phenylalanine contribute to the intrinsic fluorescence when excited at 280 nm. The intrinsic fluorescence in proteins is strongly dependent on the local environment of these fluorophores. A shift in the fluorescence maximum to a longer wavelength is observed when the environment changes from non-polar to polar. Antibodies, which have tryptophan residues in a relatively non-polar environment in the native state, have a fluorescence maximum at 337 nm which is shifted to 350 nm upon unfolding of the protein. The intrinsic fluorescence of reconstituted antibody powder formulations were measured using a SPEX-fluoromax fluorescence spectrophotometer. The samples were excited at 280 nm and fluorescence maxima measured. FIG. **3** shows fluorescence scans of reconstituted IgG:mannitol:citrate (70:20:10), IgG:PVP:citrate (70:20:10) and 100% IgG powders and a solution of IgG which had been unfolded by the denaturant guanidinium HCL. The scans of the reconstituted powder formulations show no shift in wavelength, indicating no unfolding of antibody. In contrast, the scan of the denatured IgG solution shows a wavelength shift indicative of unfolding.

UV turbidity assay

The UV turbidity assay was carried out to monitor physical stability of the protein and also to determine the yield upon reconstitution of the dry-powder in water. Suitable formulations must reconstitute in water without turbidity. The absorbance at 400 nm is a measure of the extent of aggregation (insoluble aggregates) in the solution. FIG. **4** shows that no aggregation was seen with an IgG:mannitol:citrate (70:20:10) formulation.

Dynamic Light Scattering (DLS)

Dynamic Light Scattering (DLS) measures light scattered from particles based on Brownian motion, the interaction of particles with solvent molecules. The instrument (Malvern Instruments) detects the fluctuation of light intensity using a digital correlator. The correlation functions are fitted into an analytical program to calculate the particle size distribution. DLS was used to measure soluble aggregates in reconstituted antibody powder formulations. Samples of individual blister packs were combined to give approximately 2–4 mg of powder per ml. These samples were reconstituted in water and centrifuged at 10,000 rpm for 5 minutes to remove any dust particles, which interfere with readings. The samples were decanted and placed in a cylindrical quartz cell for analysis. A semi-quantitative method of particle size determination by light intensity was developed, using a multimodal method of data analysis supplied by Malvern Instruments. Measurements were taken at a range of angles from 75° to 120°. Larger particles are more easily detectable at smaller angles, while smaller particles are more readily detected at larger angles. The IgG monomer has been reported to have a diameter of 11 nm, as measured by DLS (Singh et al., Biopolymers 31: 1387–1396 (1991)).

SEC-HPLC

SEC-HPLC was used to measure soluble aggregates in reconstituted antibody powder formulations. An HPLC system equipped with a TSK-3000 column was used. The mobile phase consisted of 0.05M $KH_2PO_4$ buffer (pH 6.8), 0.1 M KCl and 0.0015 M $NaN_3$. The assay results were obtained by determining the relative area for each peak in the chromatogram for spray dried material relative to lyophilized material. Preferred formulations showed less than 5% aggregates by SEC-HPLC.

SDS-PAGE

Silver stain SDS-PAGE analysis was used to evaluate reconstituted powder formulations for covalent aggregates of the antibodies and to confirm SEC-HPLC methods. SDS-PAGE analysis was carried out using a Pharmacia Phast system consisting of discontinuous 8 to 25% gradient Phast gels. Spray dried antibody powder formulations were reconstituted with purified water, then further diluted with SDS-PAGE buffer to an antibody concentration of 1 mg/ml. Samples were incubated in 10% SDS non-reducing sample buffer at 37° C. for 30 minutes, then 4 μg samples were loaded into each well. The gels were run and silver stained using Pharmacia standard procedures for 8 to 25% SDS-PAGE gels.

Formulation preparation:

A 100% formulation of antibody was prepared by dissolving 5 mg of IgG in 1.0 ml of deionized water. The pH of the protein solution was measured to be about 6.5.

A 90% formulation of antibody was prepared by dissolving 4.5 mg of IgG in 1.0 ml of 2mM citrate buffer. The pH of the protein solution was measured, and was generally found to be about 6.5.

A 70% formulation of antibody was prepared by dissolving 3.5 mg of antibody and 1 mg/ml of excipient in 1.0 ml of 2 mM citrate buffer. The pH of the protein solution was measured to be about 6.5.

The various classes of excipients used were as follows:

Sugar excipients: sucrose, lactose, mannitol, raffinose and trehalose.

Polymeric excipients: Ficoll and PVP.

Protein excipients: HSA.

Powder processing:

Dry powders of the above formulations were produced by spray drying using a Buchi Spray Dryer using the following parameters:

Temperature of the solution 4–6° C.

Inlet temperature 98–105° C.

Feed rate 5.0 ml/min.

Outlet temperature 64–67° C.

Atomizer pressure 40 psi

Cyclone coolant temperature 30° C.

Once the aqueous mixture was consumed, the outlet temperature was maintained at 67° C. for 10 minutes by slowly decreasing the inlet temperature to provide secondary drying.

Process suitability was assessed by evaluating the antibody powders for moisture content, particle size distribution, delivered dose, aerosol efficiency and antibody integrity.

Before preparing the antibody powders, solution formulations were analyzed to demonstrate that the protein is stable in solution over the process period during spray-drying. The antibody solutions were atomized to investigate protein aggregation in the high shear of the pneumatic atomizer. The protein stability was assayed by UV and SEC-HPLC. The absence of insoluble protein aggregates as determined by UV in the 350–400 nm region of the spectrum indicated that antibody did not aggregate or precipitate during atomization.

## EXAMPLE 1

### 100% IgG Formulation For Pulmonary Delivery

Bulk lyophilized IgG was formulated and spray dried as described above. The 100% IgG dry powder was analyzed

for moisture as described above and found to contain 2.5–3.5% moisture.

The particle size distribution was measured by centrifugal sedimentation as described above and was determined to be 1.3 μm to 1.5 μm MMD. The delivered dose of the IgG powder composition was measured as described above and was determined to be 60 to 62% of the total powder (5.0 mg) loaded into the device. The aerosol particle size distribution, measured as described above was determined to be 4.1 μm MMAD, with 61.6% of the particles <5.0 μm in diameter.

The antibody integrity was assayed by SEC-HPLC and dynamic light scattering (DLS) as described above. The powder did not reconstitute easily in water (47% reconstitution), so no UV measurement was possible. The mean diameter of particles was found to be 18 nm by DLS. The size distribution indicated the presence of a small population of soluble aggregates. We found 7.5% soluble aggregates by SEC-HPLC in the reconstituted spray dried sample. ATR FT-IR and intrinsic fluorescence assays, performed as described above, indicated no conformational change in antibody from spray drying. SDS-PAGE results showed the presence of covalent aggregates.

## EXAMPLE 2

### 90% IgG Formulation For Pulmonary Delivery

Bulk lyophilized IgG was formulated and spray dried as described above. The 90% IgG dry powder composition contained 90% IgG and 10% citrate. The formulation contained 2–2.5% moisture.

The particle size distribution was determined by centrifugal sedimentation to be 1.3 μm to 2.0 μm MMD, with 98% of the particles less than 5 μm. The delivered dose of the IgG powder was determined to be 46% of the total powder (5.0 mg) loaded into the device. The aerosol particle size distribution was determined to be 1.5 μm MMAD, with 99% of the particles <5.0 μm in diameter.

The powder gave about 66% reconstitution in water. The antibody integrity was assayed by SEC-HPLC. About 15% of soluble aggregates was detected in the reconstituted sample after spray-drying. The fluorescence maximum for the reconstituted sample was around 337 nm, indicating no alteration in antibody conformation after spray drying.

## EXAMPLE 3

### 70% IgG Formulation Containing Carbohydrate Excipient

Bulk lyophilized IgG was formulated and spray dried as described above. A 70% formulation was achieved by dissolving 3.5 mg of IgG and 1 mg sucrose in 1.0 ml of 2 mM citrate buffer. The 70% IgG dry powder composition contained 70% IgG, 20% sucrose and 10% citrate. The pH of the resulting solution was determined to be about 6.5. The formulation contained 2–2.5% moisture.

The particle size distribution was determined by centrifugal sedimentation to be 1.0 μm to 1.5 μm MMD, with 97% of the particles less than 5 μm. The delivered dose of the IgG powder was determined to be 45 to 52% of the total powder (5.0 mg) loaded into the device. The aerosol particle size distribution was determined to be 3.2 μm MMAD, with 67% of the particles <5.0 μm in diameter and 45% under 3.3 μm.

The antibody integrity was assayed by UV and SEC-HPLC. The powder reconstituted well, with complete recovery of antibody and absence of aggregates as shown by UV. About 3–4% of soluble aggregates were detected by SEC-HPLC in the reconstituted sample after spray-drying. The fluorescence maximum for the reconstituted sample was around 337 nm, indicating no alteration in antibody conformation after spray drying.

## EXAMPLE 4

### 70% IgG Formation Containing Polymeric Excipient

Bulk lyophilized IgG was formulated and spray dried as described above. A 70% formulation was achieved by dissolving 3.5 mg of IgG and 1 mg of PVP in 1.0 ml of 2 mM citrate buffer. The pH of the protein solution was measured to be at 6.7. The 70% IgG dry powder composition contained 70% IgG, 20% PVP and 10% citrate. The formulation contained 1.5–2% moisture.

The particle size distribution was determined by centrifugal sedimentation to be 1.7 μm to 2.0 μm MMD, with 97% of the particles less than 5 μm. The delivered dose of the IgG powder was determined to be 62% of the total powder (5.0 mg) loaded into the device. The aerosol particle size distribution showed that 41% of the particles were <3.3 μm in diameter.

Antibody integrity was assayed by UV. The absence of insoluble protein aggregates as determined by UV in the 350–400 nm region of the spectrum indicated that IgG did not aggregate or precipitate during spray-drying. The fluorescence maximum for the reconstituted sample was around 337 nm, indicating no alteration in antibody conformation after spray drying.

## EXAMPLE 5

### 70% IgG Formulation With Protein Excipient

Bulk lyophilized IgG was formulated and spray dried as described above. A 70% formulation was achieved by dissolving 3.5 mg of IgG and 1 mg HSA (Miles-Pentex) in 1.0 ml of 2 mM citrate buffer. The pH of the protein solution was measured to be at 6.2. The 70% IgG dry powder composition contained 70% IgG, 20% HSA and 10% citrate. The formulation contained 2.8–3.3% moisture.

The particle size distribution was determined by centrifugal sedimentation to be 1.3–1.5 μm MMD, with 100% of the particles less than 5 μm. The delivered dose of the IgG powder was determined to be 70 to 75% of the total powder (5.0 mg) loaded into the device. The aerosol particle size distribution was determined to be 3.8 μm MMAD, with 68% of the particles <5.0 μm in diameter.

Antibody integrity was assayed by UV and SEC-HPLC. The powder did not reconstitute easily, with only about 35% recovered after reconstitution based on UV analysis. About 9% of soluble aggregates was determined by SEC-HPLC. No fluorescence determination was performed, due to interference that would result from the presence of another protein, i.e., HSA.

## EXAMPLE 6

### 90% IgG Formulation With Surfactant Excipient

Bulk lyophilized IgG was formulated and spray dried as described above. A 90% formulation was achieved by dissolving 4.5 mg of IgG in 1.0 ml of 2 mM citrate buffer containing 0.05% BAC. The pH of the protein solution was measured to be at 6.5. The above 90% IgG dry powder

composition contained 89.95%lo IgG, 0.05% BAC and 10% citrate. The formulation contained 3–3.5% moisture.

The particle size distribution was determined by centrifugal sedimentation to be 1.4 μm MMD, with 100% of the particles less than 5 μm. The delivered dose of the IgG powder was determined to be 45% of the total powder (5.0 mg) loaded into the device. The aerosol particle size distribution was determined to be 3.6 μm MMAD, with 69% of the particles <5.0 μm in diameter.

The IgG-surfactant powder gave a turbid-looking solution upon reconstitution.

## EXAMPLE 7

### 100% IgA Formulation

IgA obtained from Sigma was preformulated in 10 mM Tris and 100 mM NaCl, pH 7.4. It was dialyzed against de-ionized water overnight at 4° C. The dialyzed protein was concentrated using a filtron unit to 5 mg/ml. The pH of the resulting solution was determined to be 6.9. The solution was spray dried as described above. The 100% IgA dry powder contained 3–3.5% moisture.

The particle size distribution was determined by centrifugal sedimentation to be 1.22 μm MMD, with 97% of the particles less than 5 μm. The delivered dose of the IgA powder was determined to be 50–52% of the total powder (5.0 mg) loaded into the device.

Antibody integrity was assayed by UV and dynamic light scattering. The absence of insoluble protein aggregates as determined by UV in the 350–400 nm region of the spectrum indicated that IgA did not aggregate or precipitate during atomization.

## EXAMPLE 8

### 70% IgA Formulation Containing Protein Excipient

IgA obtained from Sigma was preformulated in 10 mM Tris and 100 mM NaCl, pH 8.0. It was dialyzed against 2 mM citrate buffer, pH 6.5 overnight at 4° C. 40 mg of HSA (Miles Pentex) was dissolved in 30 ml of dialyzed IgA solution (4.16 mg/ml) and the resulting solution spray dried as described above. The 70% IgA dry powder composition contained 70% IgA, 20% HSA and 10% citrate. The formulation contained 2.6% moisture.

The particle size distribution was determined by centrifugal sedimentation to be 1.22 μm MMD, with 97% of the particles less than 5 μm. The delivered dose of the IgA powder was determined to be 65% of the total powder (5.0 mg) loaded into the device.

## EXAMPLE 9

### 70% IgG Formulation Containing Carbohydrate Excipient

Bulk lyophilized IgG was formulated and spray dried as described above. A 70% formulation was achieved by dissolving 3.5 mg of IgG and 1 mg mannitol in 1.0 ml of 2 mM citrate buffer. The 70% IgG dry powder composition contained 70% IgG, 20% mannitol and 10% citrate. The pH of the resulting solution was determined to be about 6.5. The formulation contained 1.7% moisture.

The particle size was determined by centrifugal sedimentation to be 1.9 μm MMD, with 100% of the particles less than 5 μm. The delivered dose of the IgG powder was determined to be 53%±4% of the total powder (5.0 mg) loaded into the device. The aerosol particle size distribution showed that 45% of the particles were <3.3 μm in diameter.

The antibody integrity was assayed by UV and SEC-HPLC. The powder reconstituted well, with complete recovery of antibody and absence of aggregates as shown by UV. About 3–4% of soluble aggregates were detected by SEC-HPLC in the reconstituted sample after spray-drying. The fluorescence maximum for the reconstituted sample was around 337 nm, indicating no alteration in antibody conformation after spray drying. SDS-PAGE results showed the presence of covalent aggregates.

## EXAMPLE 10

### 70% IgG Formulation Containing Polymeric Excipient

Bulk lyophilized IgG was formulated and spray dried as described above. A 70% formulation was achieved by dissolving 3.5 mg of IgG and 1 mg of Ficoll-4000 in 1.0 ml of 2 mM citrate buffer. The pH of the protein solution was measured to be at 6.7. The 70% IgG dry powder composition contained 70% IgG, 20% Ficoll and 10% citrate. The formulation contained 1.9% moisture.

The particle size distribution was determined by centrifugal sedimentation to be 1.78 μm MMD, with 100% of the particles less than 5 μm. The delivered dose of the IgG powder was determined to be 62% of the total powder (5.0 mg) loaded into the device.

## EXAMPLE 11

### Effect Of Excipients On DDE Of Spray Dried IgG Powders

The delivered dose efficiency (DDE) of the spray dried IgG powders varied considerably, depending on the type of excipient used. The 100% IgG powders showed a delivered dose efficiency of about 60%. The use of sugars as excipients decreased the delivered dose efficiency to about 40–55%. Addition of polymeric excipients such as Ficoll and PVP did not alter the delivered dose efficiency (with respect to the 100% IgG). Results are presented in Table 1.

HSA is a commonly used protein excipient for parenteral delivery. An increase in the delivery efficiency of powders of small molecules and proteins upon addition of HSA has been observed. Addition of HSA to the solutions used to form spray dried IgG powders resulted in an increase in the DDE, although the increase seemed to be independent of the HSA concentration (5–20%). The mass median aerodynamic diameters (MAD) ranged from 2.5–4 μm for all powders tested.

TABLE 1

Effect of Excipients on DDE of Spray Dried IgG Powders

| Powder Formulation | Delivered Dose Efficiency | Relative Standard Deviation |
|---|---|---|
| 100% IgG | 60 | 8 |
| 100% IgG | 62 | 1 |
| 90% IgG, 10% Citrate | 37 | 7 |
| 90% IgG, 10% Citrate | 44 | 9 |
| 70% IgG, 20% Sucrose, 10% Citrate | 53 | 6 |
| 40% IgG, 20% Sucrose, 10% Citrate | 52 | 5 |
| 70% IgG, 20% Mannitol, 10% Citrate | 53 | 4 |
| 70% IgG, 20% Trehalose, 10% Citrate | 49 | 7 |

TABLE 1-continued

Effect of Excipients on DDE of Spray Dried IgG Powders

| Powder Formulation | Delivered Dose Efficiency | Relative Standard Deviation |
|---|---|---|
| 70% IgG, 20% PVP, 10% Citrate | 63 | 3 |
| 70% IgG, 30% Citrate | 48 | 18 |
| 70% IgG, 20% Lactose, 10% Citrate | 48 | 5 |
| 70% IgG, 20% Raffinose, 10% Citrate | 42 | 4 |
| 70% IgG, 20% Ficoll 400, 10% Citrate | 62 | 6 |
| 70% IgG, 20% HSA, 10% Citrate | 70 | 5 |
| 70% IgG, 30% HSA | 75 | 2 |
| 70% IgG, 20% HSA, 10% Citrate | 71 | 6 |
| 70% IgG, 20% HSA, 10% Citrate | 73 | 10 |
| 85% IgG, 15% HSA | 79 | 5 |
| 95% IgG, 5% HSA | 80 | 1 |
| 85% IgG, 15% HSA | 77 | 4 |
| 85% IgG, 15% HSA | 79 | 2 |
| 85% IgG, 15% HSA | 71 | 7 |
| 95% IgG, 5% HSA | 76 | 5 |

## EXAMPLE 12

### Rugosity Of Antibody Powders

The rugosity of certain dry powder antibody compositions of the present invention is presented in Table 2. Results showed that all antibody powders and a powder composed of 100% HSA had rugosity of at least 2.

TABLE 2

Rugosity of Antibody Powders

| Powder Formulation | Rugosity | DDE |
|---|---|---|
| 100% IgG | 2.02 | 60 |
| IgG + Citrate | 2.40 | 51 |
| 100% HSA | 3.37 | 81.4 |
| IgG + HSA | 2.93 | 75.3 |

Modification of the above-described modes of carrying out the various embodiments of this invention will be apparent to those skilled in the art following the teachings of this invention as set forth herein. The examples described above are not limiting, but are merely exemplary of this invention, the scope of which is defined by the following claims.

The disclosure of each publication, patent or patent application mentioned in this specification is hereby incorporated by reference to the same extent as if each individual publication, patent or patent application were specifically and individually indicated to be incorporated by reference.

We claim:

**1**. An antibody-based dry powder composition for pulmonary delivery comprising a predetermined amount of antibody which is non-aggregated and is in its native conformation in respirable dry powder particles having a particle size less than 10 micrometers, wherein said composition has a delivered dose efficiency of at least about 30%.

**2**. The composition of claim **1** which is dispersible.

**3**. The composition of claim **1** wherein the antibody is selected from the group consisting of IgG, IgA and IgM antibodies.

**4**. The composition of claim **1** which further comprises a pharmaceutically acceptable excipient or carrier.

**5**. The composition of claim **4** wherein the carrier or excipient is selected from the group consisting of sugars, alditols, organic salts, amino acids, polymers, proteins and peptides.

**6**. The composition of claim **1** wherein the antibody comprises at least about 40% by weight of the composition.

**7**. The composition of claim **1** wherein the particles in the powder have a particle size range of from about 0.4 $\mu$m to about 5 $\mu$m MMD.

**8**. The composition of claim **1** wherein the particles have an MMAD of less than about 5 $\mu$m.

**9**. The composition of claim **1** which contains less than about 10% by weight moisture.

**10**. A method for preparing the composition of claim **1** comprising:

a) mixing the antibody with a solvent to form a solution or suspension; and

b) spray drying the mixture formed in step a) under conditions which provide a respirable dry powder, wherein the antibody is non-aggregated and is in its native conformation.

**11**. The method of claim **10** further comprising the step of adding a pharmaceutically acceptable excipient or carrier prior to spray drying.

**12**. The method of claim **10** wherein the solvent is selected from the group consisting of water and ethanol.

**13**. A method of treating or preventing a condition in an animal which condition may be prevented or alleviated by an antibody, the method comprising pulmonary administration of a therapeutically effective amount of the composition of claim **1** to an animal susceptible to or suffering from the condition.

**14**. The method of claim **13** wherein the condition is selected from the group consisting of inflammation, allergy, cancer, bacterial infection and viral infection.

* * * * *

US006171799B1

(12) **United States Patent**
**Skibbens et al.**

(10) **Patent No.: US 6,171,799 B1**
(45) **Date of Patent: Jan. 9, 2001**

(54) **MONOCLONAL ANTIBODIES REACTIVE WITH DEFINED REGIONS OF THE T CELL ANTIGEN RECEPTOR**

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(73) Assignee: **Astra AB**, Södertalje (SE)

( * ) Notice: Under 35 U.S.C. 154(b), the term of this patent shall be extended for 0 days.

(21) Appl. No.: **08/450,275**

(22) Filed: **May 25, 1995**

**Related U.S. Application Data**

(62) Division of application No. 08/083,408, filed on Jun. 25, 1993, now Pat. No. 6,048,526, which is a division of application No. 07/449,692, filed on Dec. 11, 1989, now Pat. No. 5,223,426, which is a continuation-in-part of application No. 07/343,189, filed on Apr. 25, 1989, now abandoned, which is a continuation-in-part of application No. 07/284,511, filed on Dec. 15, 1988, now abandoned.

(51) **Int. Cl.**[7] ..... **G01N 33/53**

(52) **U.S. Cl.** ..... **435/7.1**; 436/503; 436/548; 436/63; 436/804; 436/811

(58) **Field of Search** ..... 424/144.1; 530/388.22, 530/388.75; 435/240.27, 172.3, 70.21, 7.1; 436/503, 548, 63, 804, 811

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| 2 197 323 | 5/1988 | (GB) . |
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(74) *Attorney, Agent, or Firm*—Banner & Witcoff, Ltd.

(57) **ABSTRACT**

The present invention relates to monoclonal antibodies which recognize defined regions of the T-cell receptor (TCR). In a specific embodiment, the invention provides monoclonal antibodies which are reactive with a constant region of the alpha chain of the TCR. In particular embodiments, the invention relates to two monoclonal antibodies, termed αF1 and αF2, which react with two different epitopes on the framework region of the α monomer of the TCR molecule. In another specific embodiment, the invention is directed to monoclonal antibodies reactive with a variable region of the beta chain of the TCR. In particular, the invention provides two monoclonal antibodies, termed W112 and 2D1, which react with β chain variable regions Vβ5.3 and Vβ8.1, respectively. In another specific embodiment, the invention is directed to monoclonal antibodies reactive with a variable region of the delta chain of the TCR. In particular, the invention provides monoclonal antibody δTCS1, isotype IgG2a. The monoclonal antibodies of the invention have value in diagnosis and therapy and are useful tools for study of the immune system.

**12 Claims, 26 Drawing Sheets**

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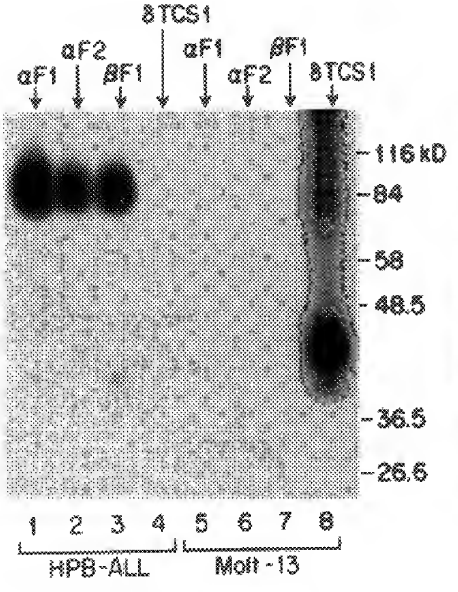
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αF1
βF1
αF1
αF2
βF1
δTCS1
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36.5
26.6
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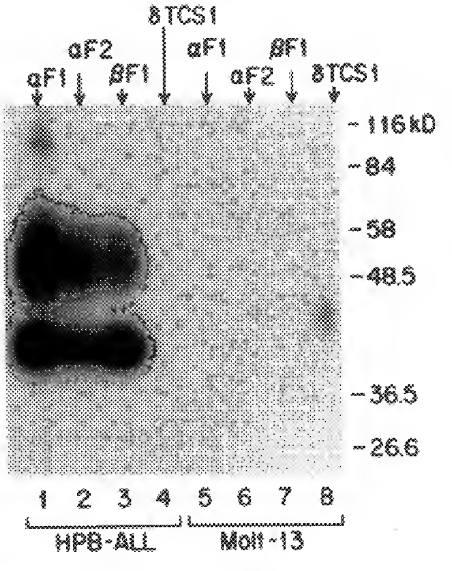
HPB-ALL
Molt-13

FIG. 1A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26.6
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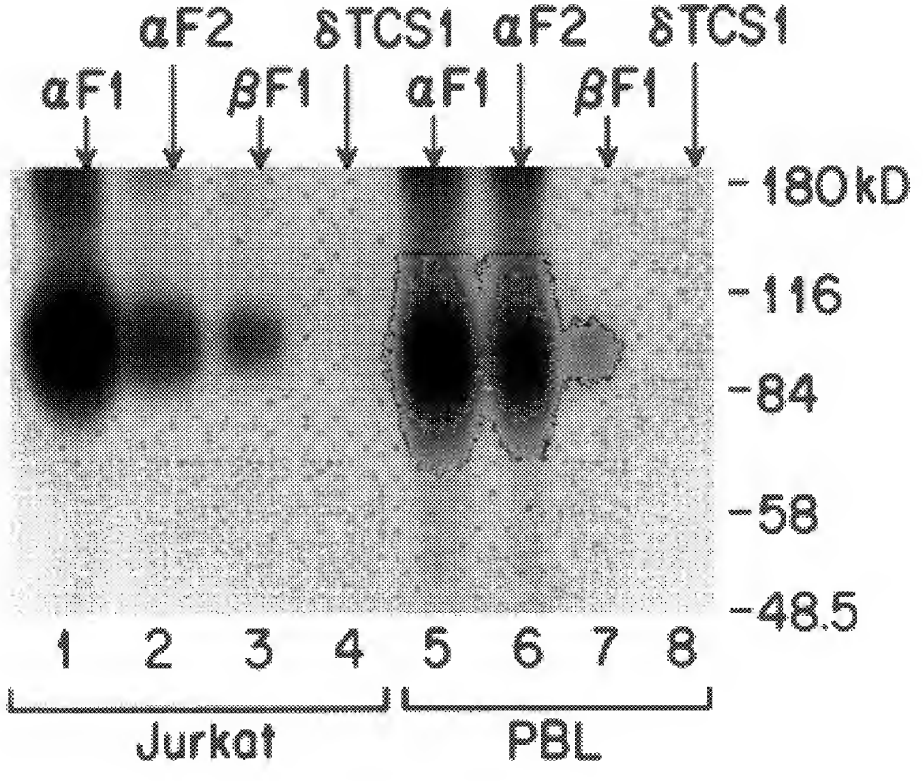
Molt-13

FIG. 1B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58
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1 2 3 4 5 6 7 8
Jurkat
PBL

FIG. 2

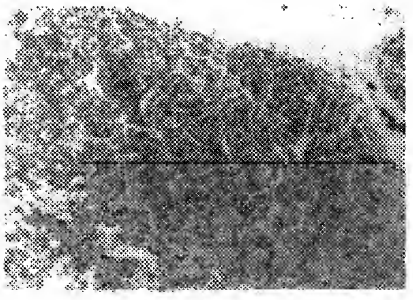


FIG. 3A

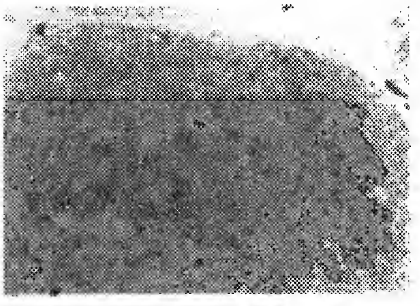


FIG. 3B

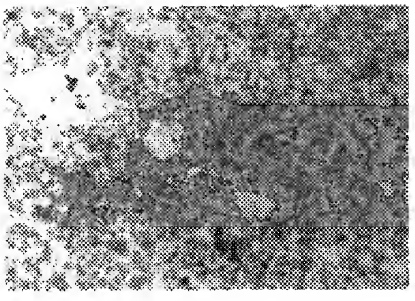


FIG. 3C

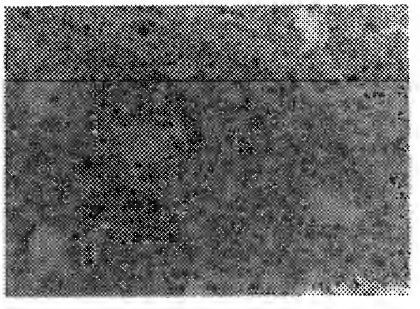


FIG. 3D

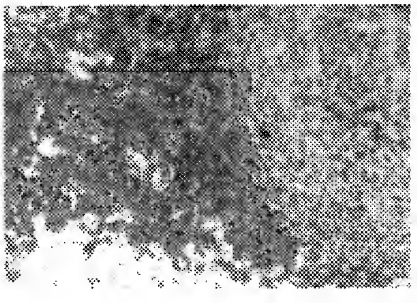


FIG. 3E

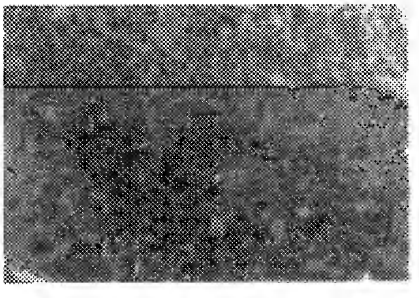
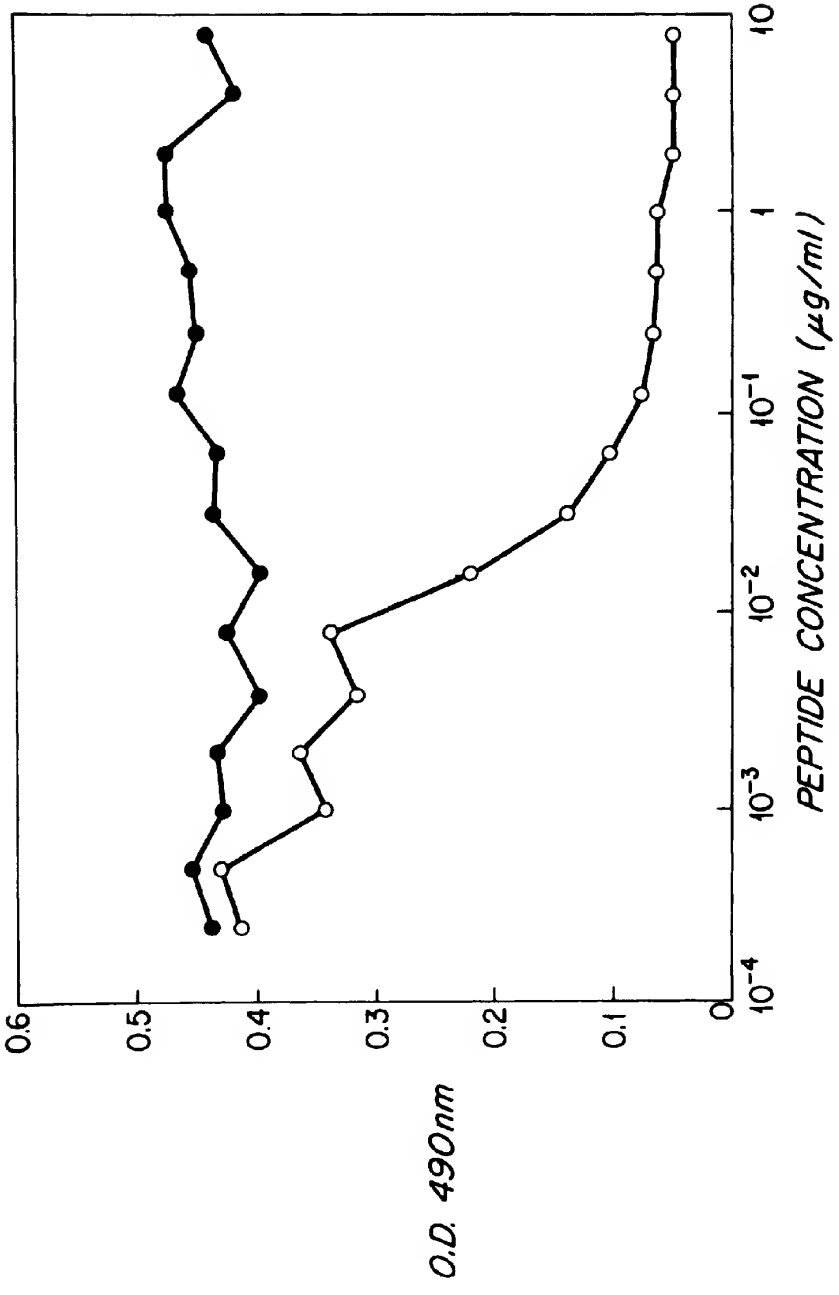


FIG. 3F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0.5
0.4
0.3
0.2
0.1
0
O.D. 490nm
$10^{-4}$
$10^{-3}$
$10^{-2}$
$10^{-1}$
1
10
PEPTIDE CONCENTRATION (μg/ml)

FIG. 4A

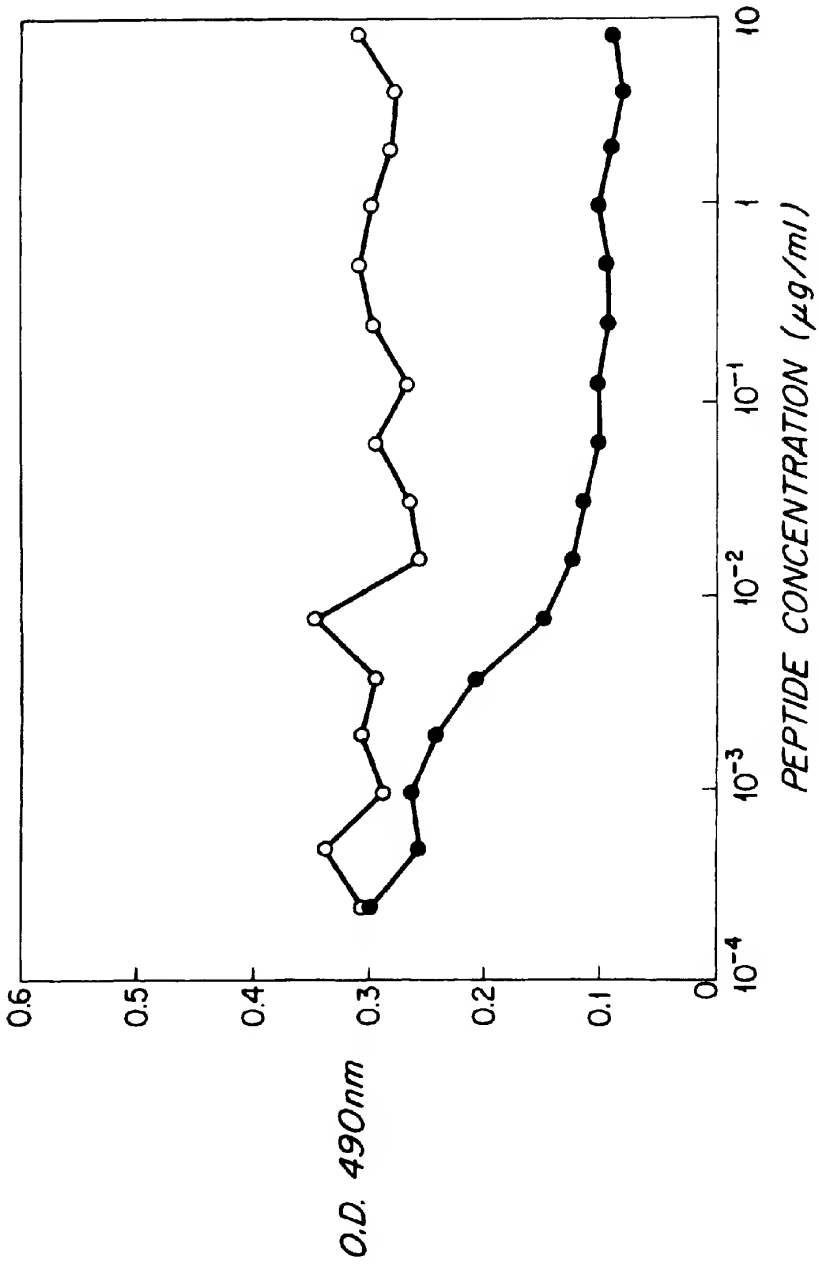
O.D. 490nm
0.6
0.5
0.4
0.3
0.2
0.1
0
$10^{-4}$
$10^{-3}$
$10^{-2}$
$10^{-1}$
1
10
PEPTIDE CONCENTRATION (μg/ml)

FIG. 4B

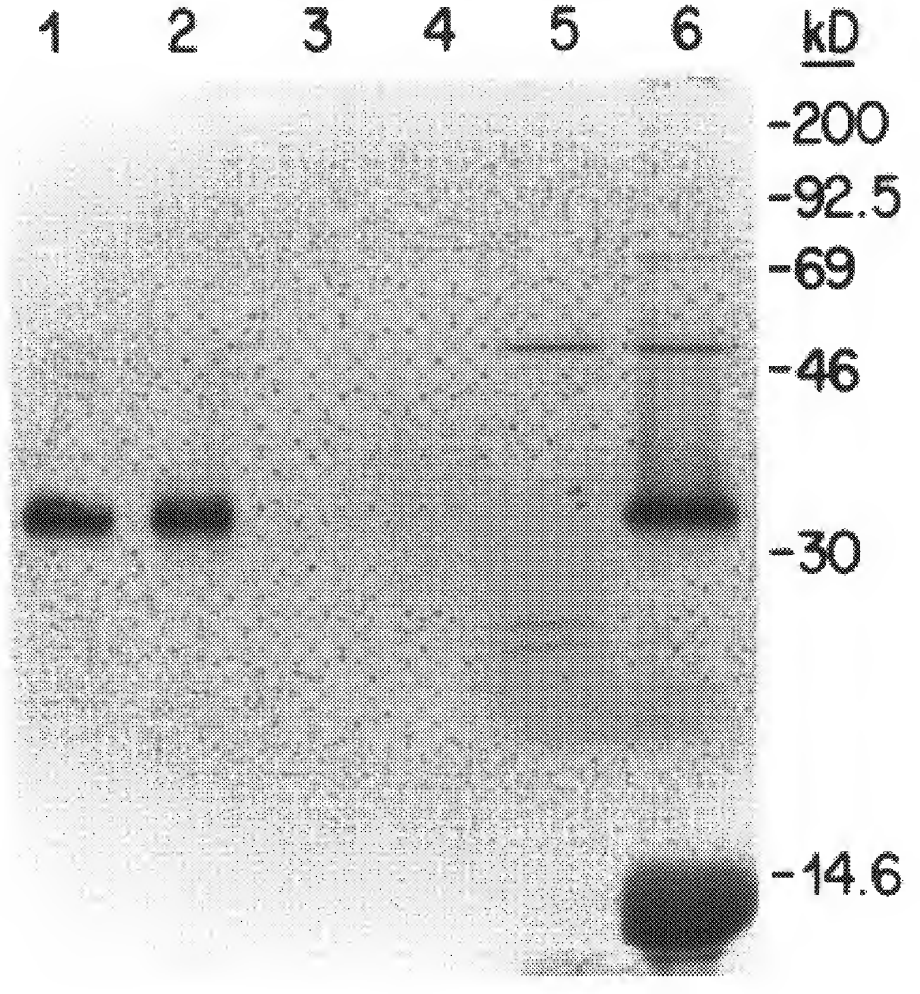


FIG. 5

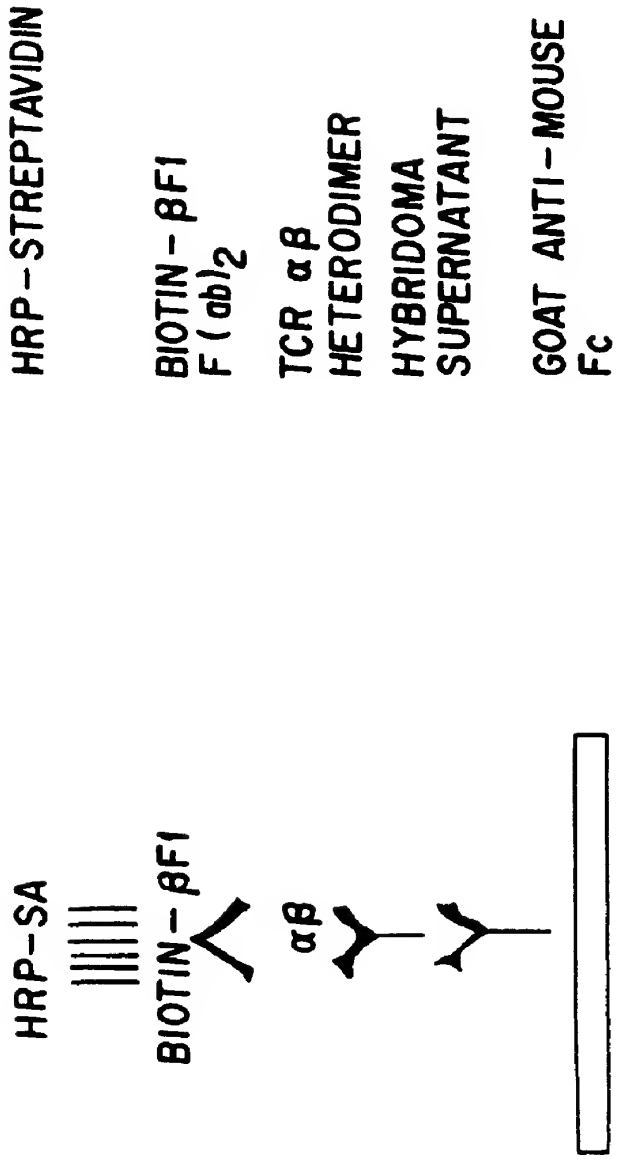
HRP-STREPTAVIDIN
BIOTIN-βF1
F(ab)2
TCR αβ
HETERODIMER
HYBRIDOMA
SUPERNATANT
GOAT ANTI-MOUSE Ig
Fc
HRP-SA
BIOTIN-βF1
αβ

FIG. 6A

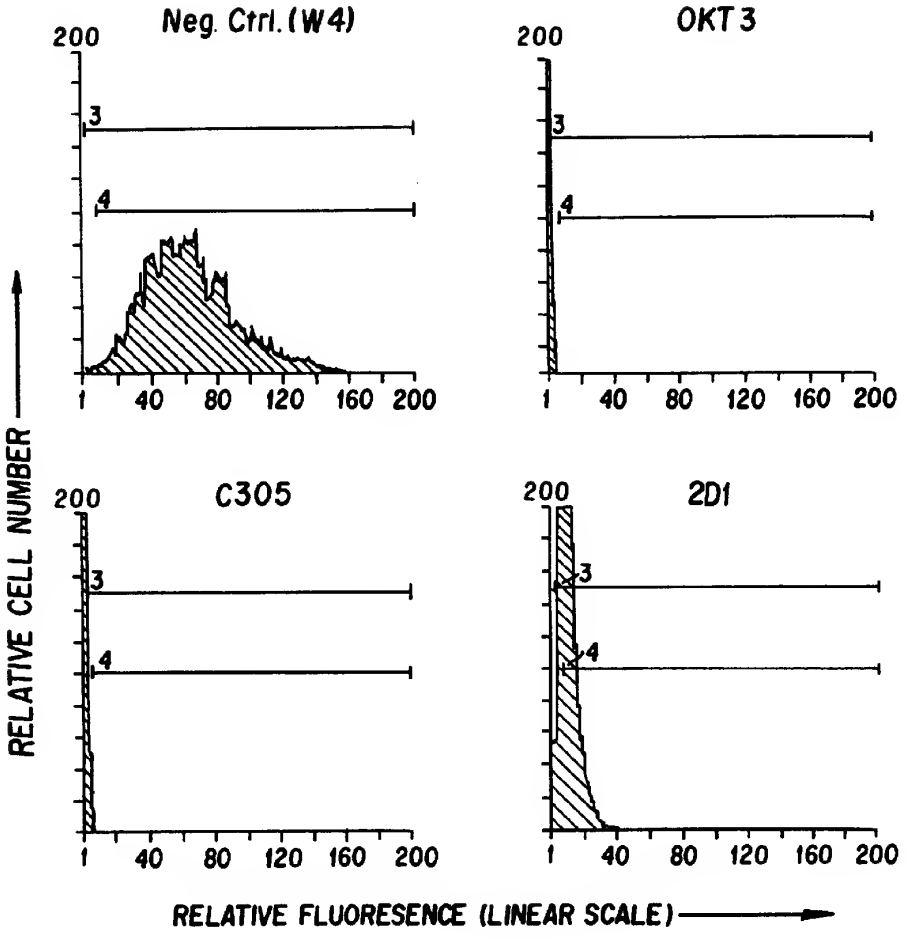
Neg. Ctrl. (W4)
OKT3
C305
2D1
200
3
4
1
40
80
120
160
200
RELATIVE CELL NUMBER
RELATIVE FLUORESCENCE (LINEAR SCALE)

FIG. 6B

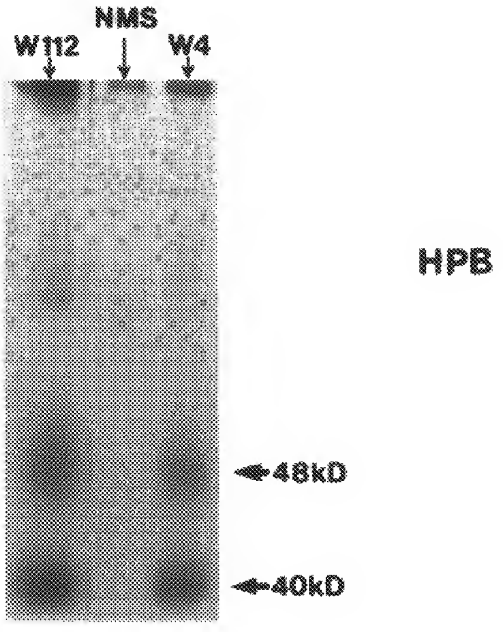


FIG. 7A

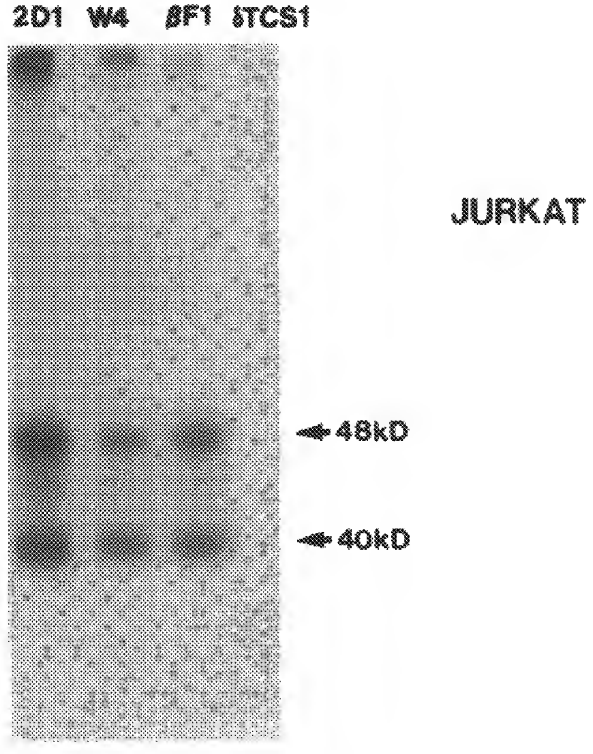


FIG. 7B

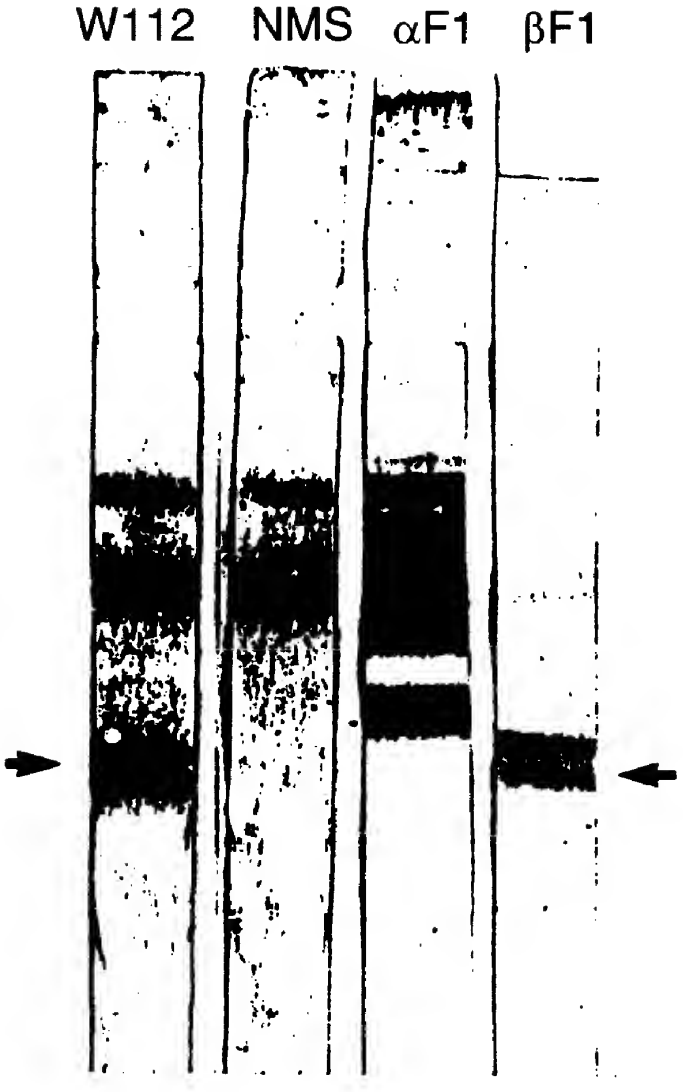


FIG. 8

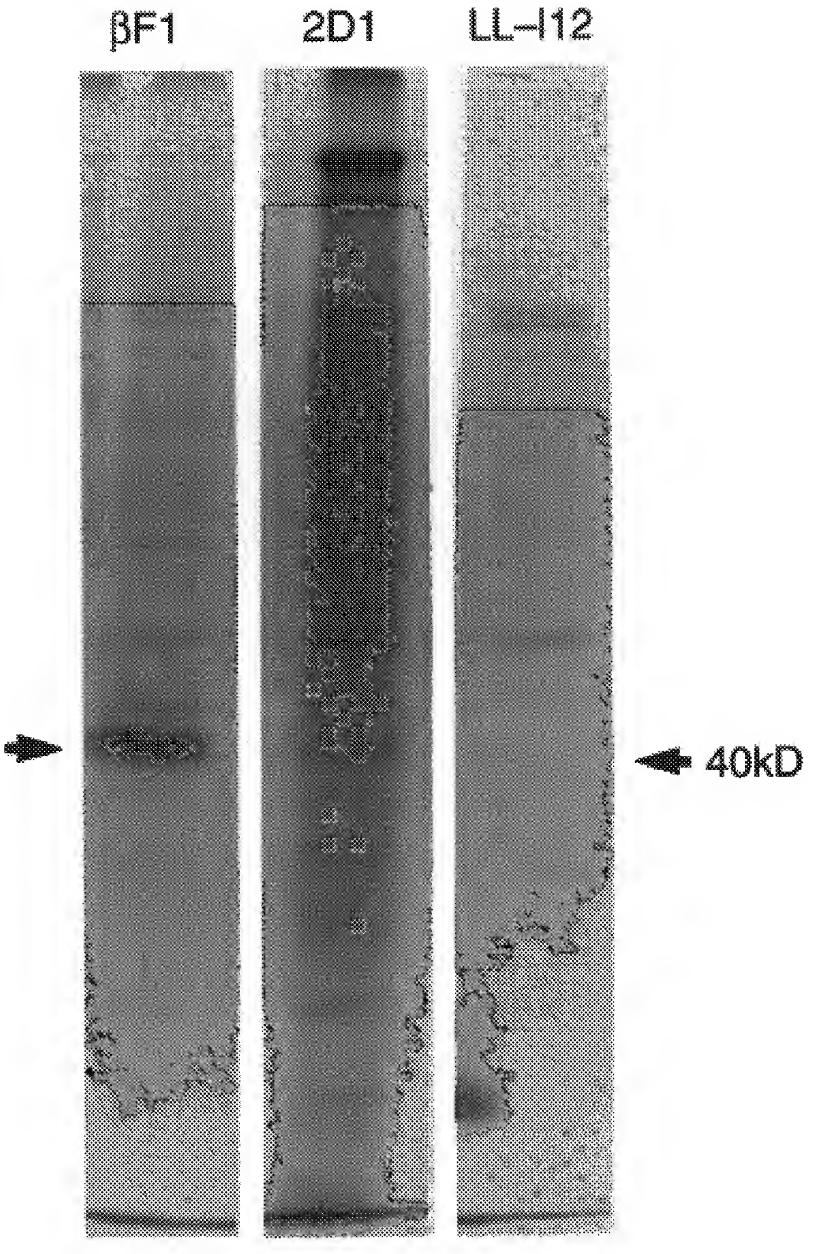


FIG. 9

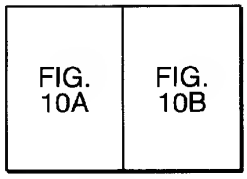


FIG. 10

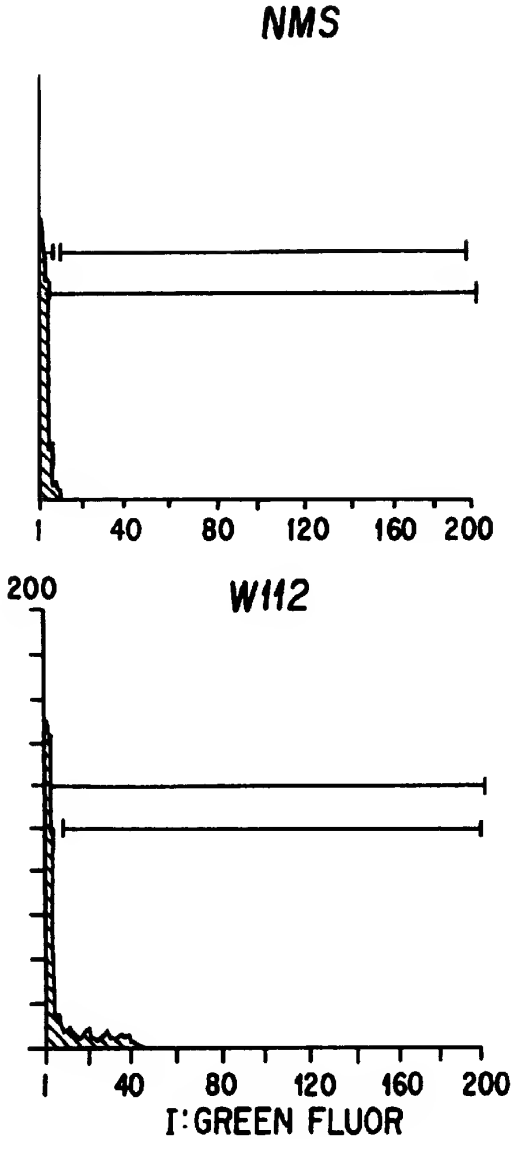


FIG. 10A

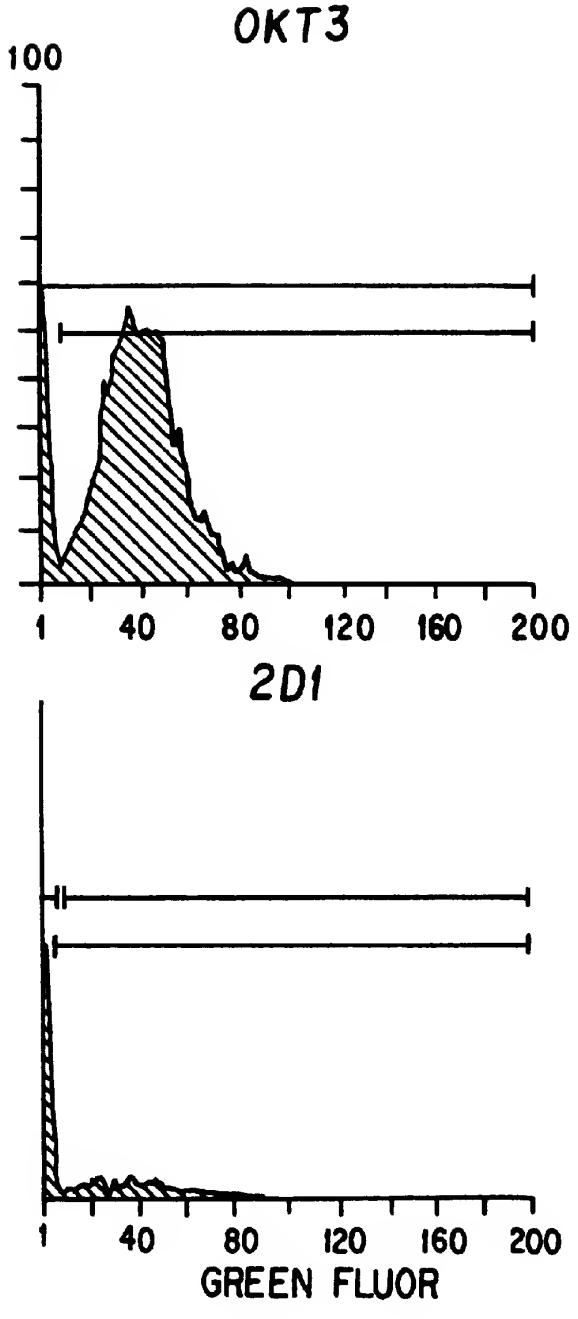


FIG. 10B

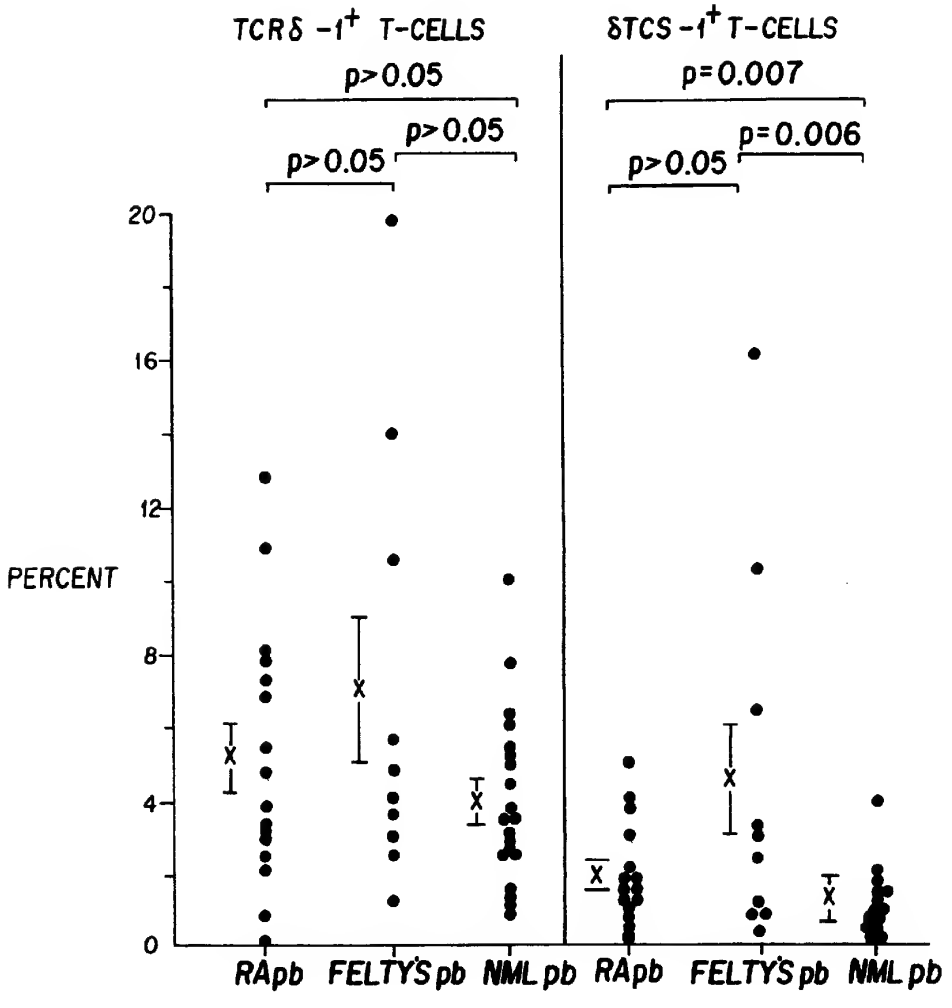
TCRδ -1+ T-CELLS
δTCS -1+ T-CELLS
p>0.05
p>0.05
p>0.05
p=0.007
p=0.006
p>0.05
PERCENT
20
16
12
8
4
0
RApb
FELTYSpb
NMLpb
RApb
FELTYSpb
NMLpb

FIG. 11

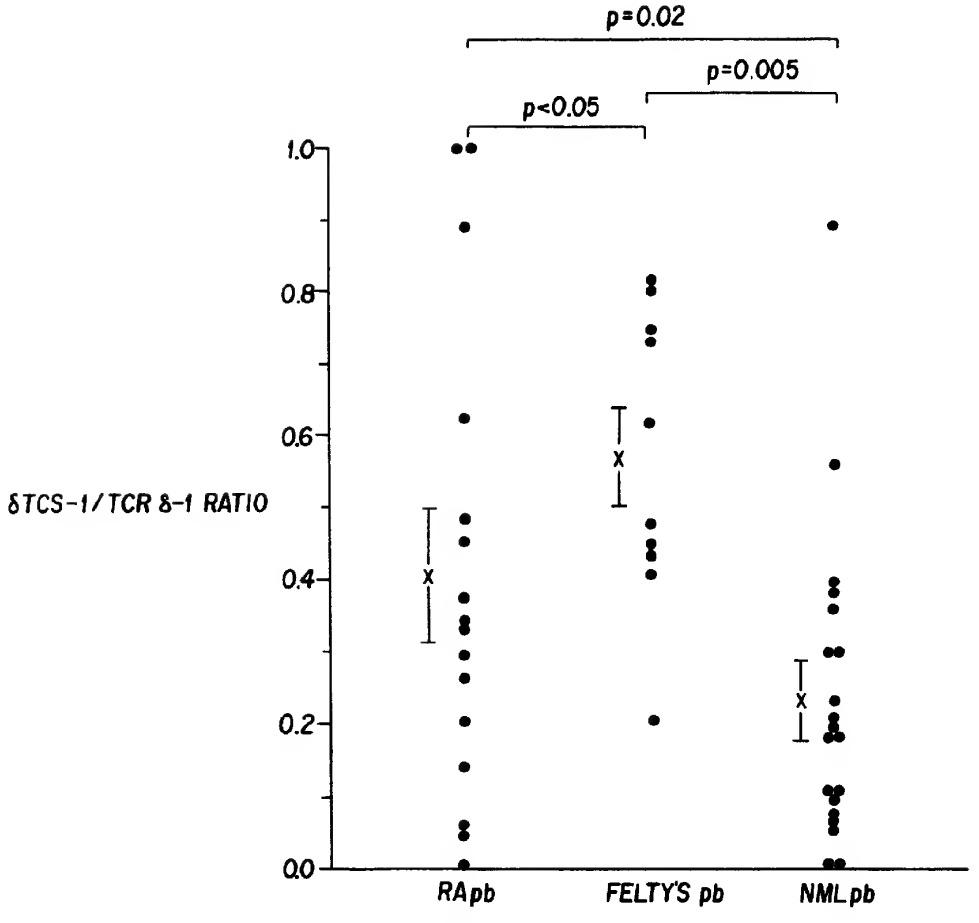
p=0.02
p=0.005
p<0.05
δTCS-1/TCR δ-1 RATIO
1.0
0.8
0.6
0.4
0.2
0.0
RApb
FELTY'S pb
NMLpb

FIG. 12

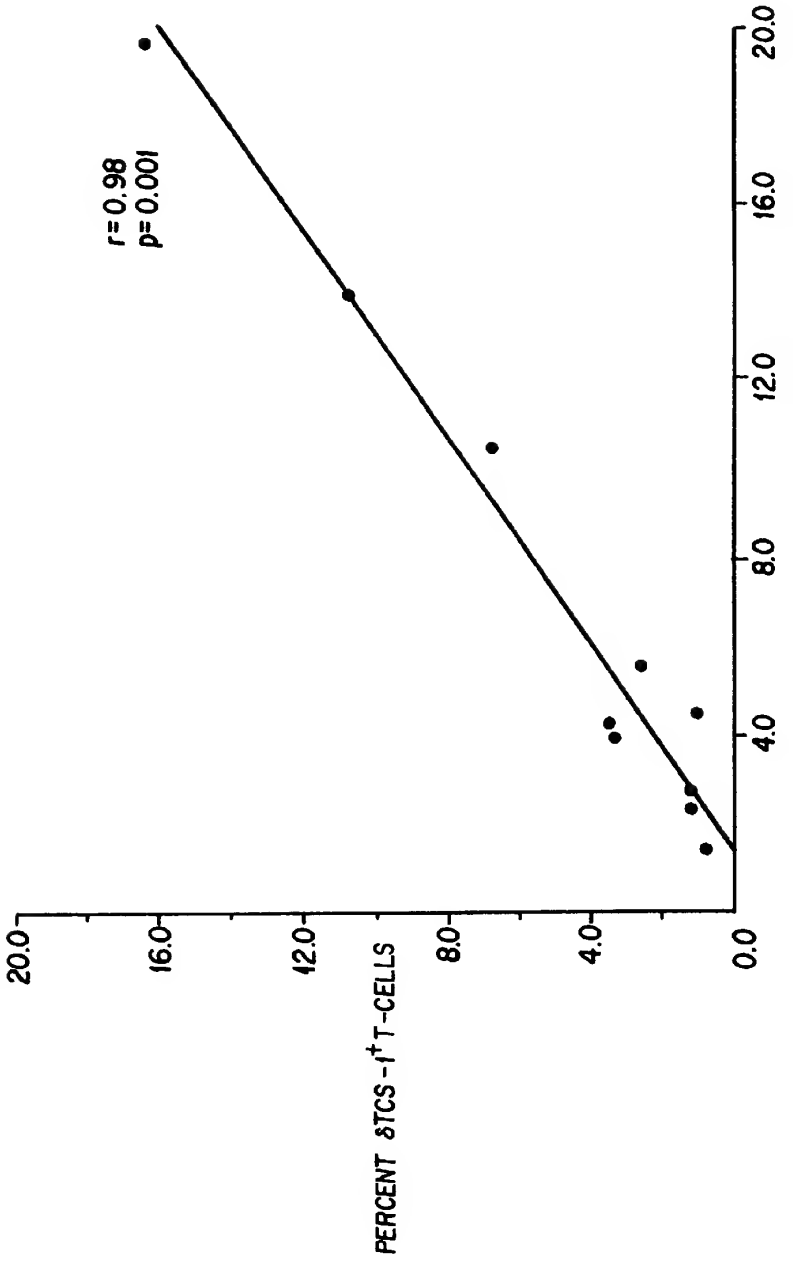
r= 0.98
p= 0.001
PERCENT δTCS -1+ T-CELLS
PERCENT TCRδ -1+ T-CELL
0.0
4.0
8.0
12.0
16.0
20.0

FIG. 13

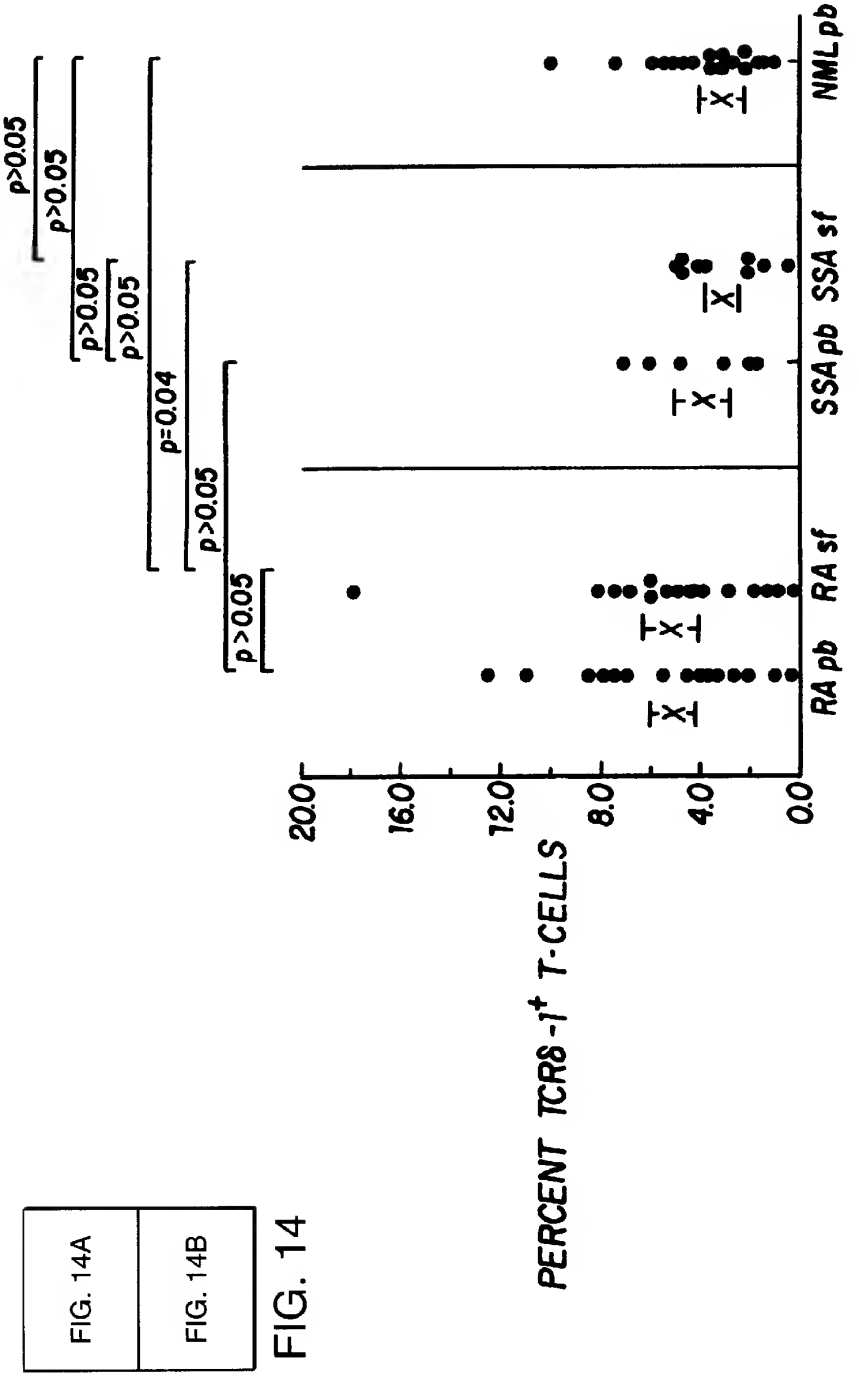


FIG. 14A

FIG. 14

| FIG. 14A |
| --- |
| FIG. 14B |

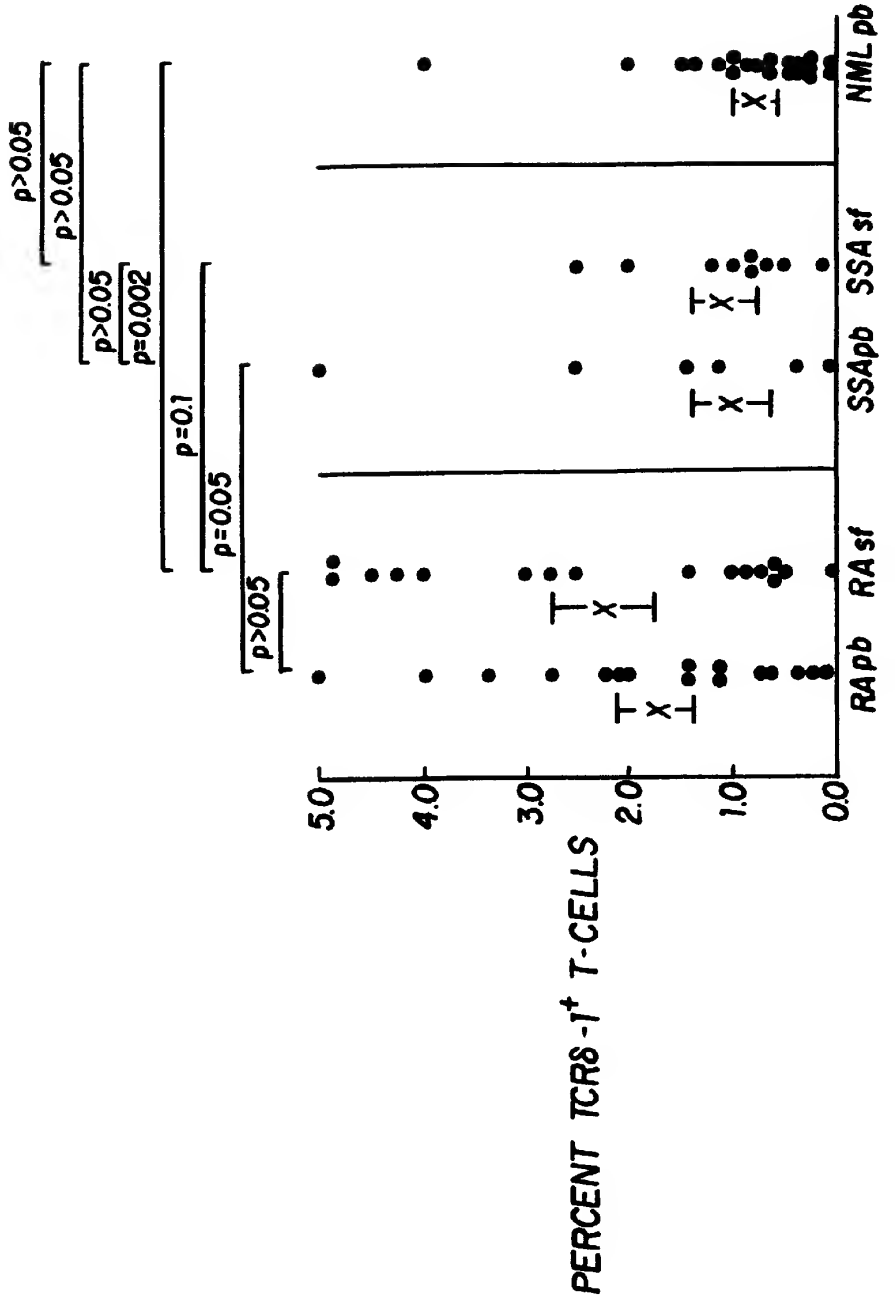
p>0.05
p>0.05
p>0.05
p=0.002
p=0.1
p=0.05
p>0.05
5.0
4.0
3.0
2.0
1.0
0.0
PERCENT TCR8-1+ T-CELLS
RApb
RA sf
SSApb
SSA sf
NML pb

FIG. 14B

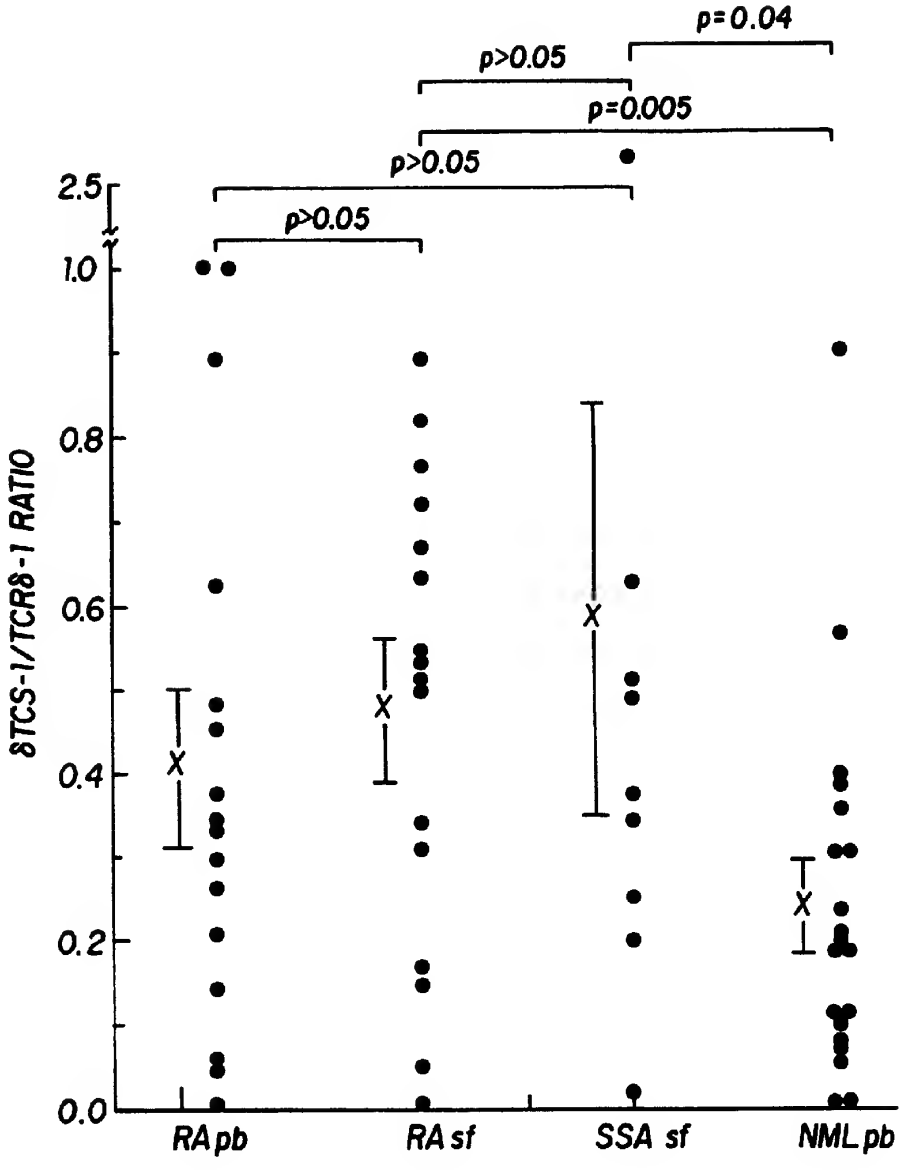
p=0.04
p>0.05
p=0.005
p>0.05
p>0.05
2.5
1.0
0.8
0.6
0.4
0.2
0.0
δTCS-1/TCRδ-1 RATIO
RA pb
RA sf
SSA sf
NML pb

FIG. 15

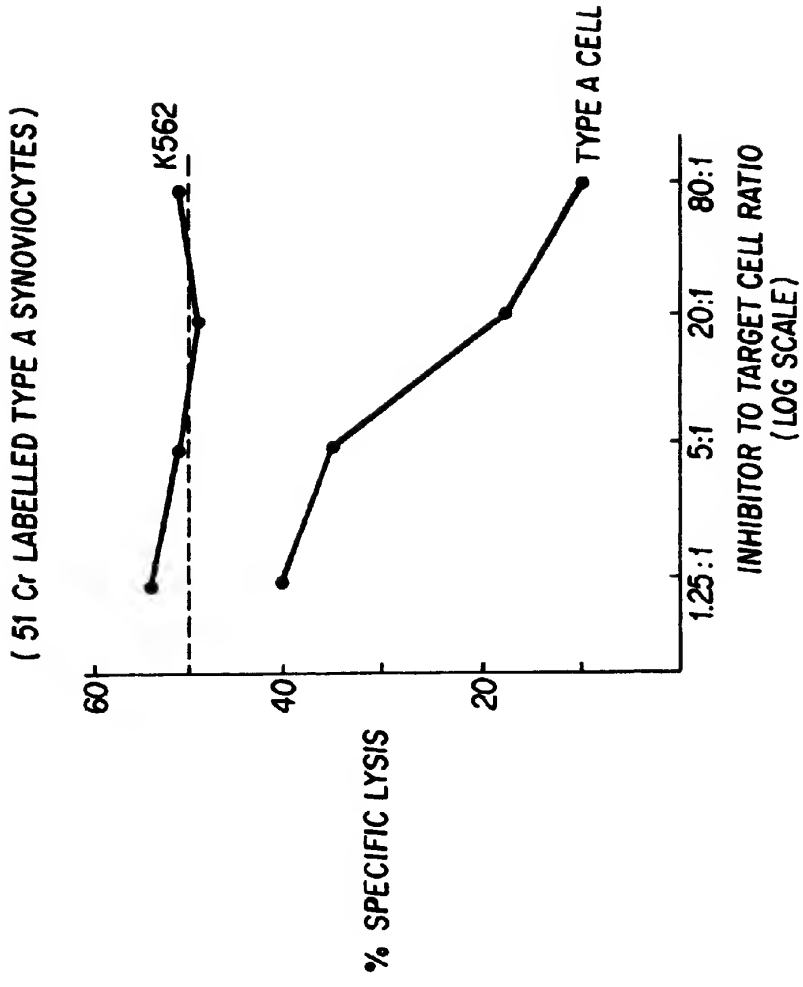
( 51 Cr LABELLED TYPE A SYNOVIOCYTES )
K562
TYPE A CELL
60
40
20
% SPECIFIC LYSIS
1.25:1
5:1
20:1
80:1
INHIBITOR TO TARGET CELL RATIO
(LOG SCALE)

FIG. 16

= active joints

x = effusions

1. FUNCTIONAL CLASS
2. A.M. STIFFNESS (MIN.)
3. JOINT COUNT
4. EFFUSION COUNT
5. LANDSBURY INDEX
6. GRIP STRENGTH
   RIGHT
   LEFT
7. SIZE / INVOLV JOINTS
8. DAMAGED JOINTS
9. DAMAGE/DURATION INDEX
10. EXTRA-ARTICULAR
    MANIFESTATIONS

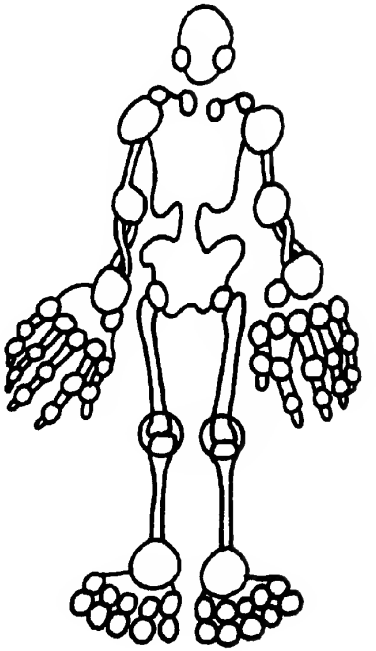

FIG. 17

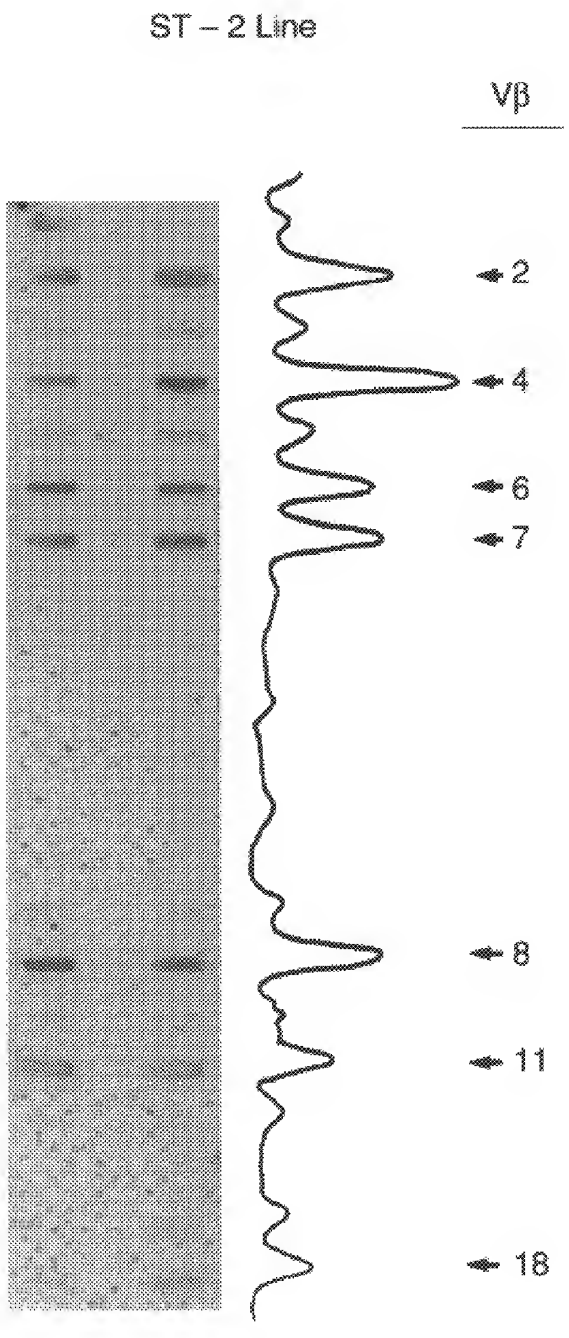


FIG. 18

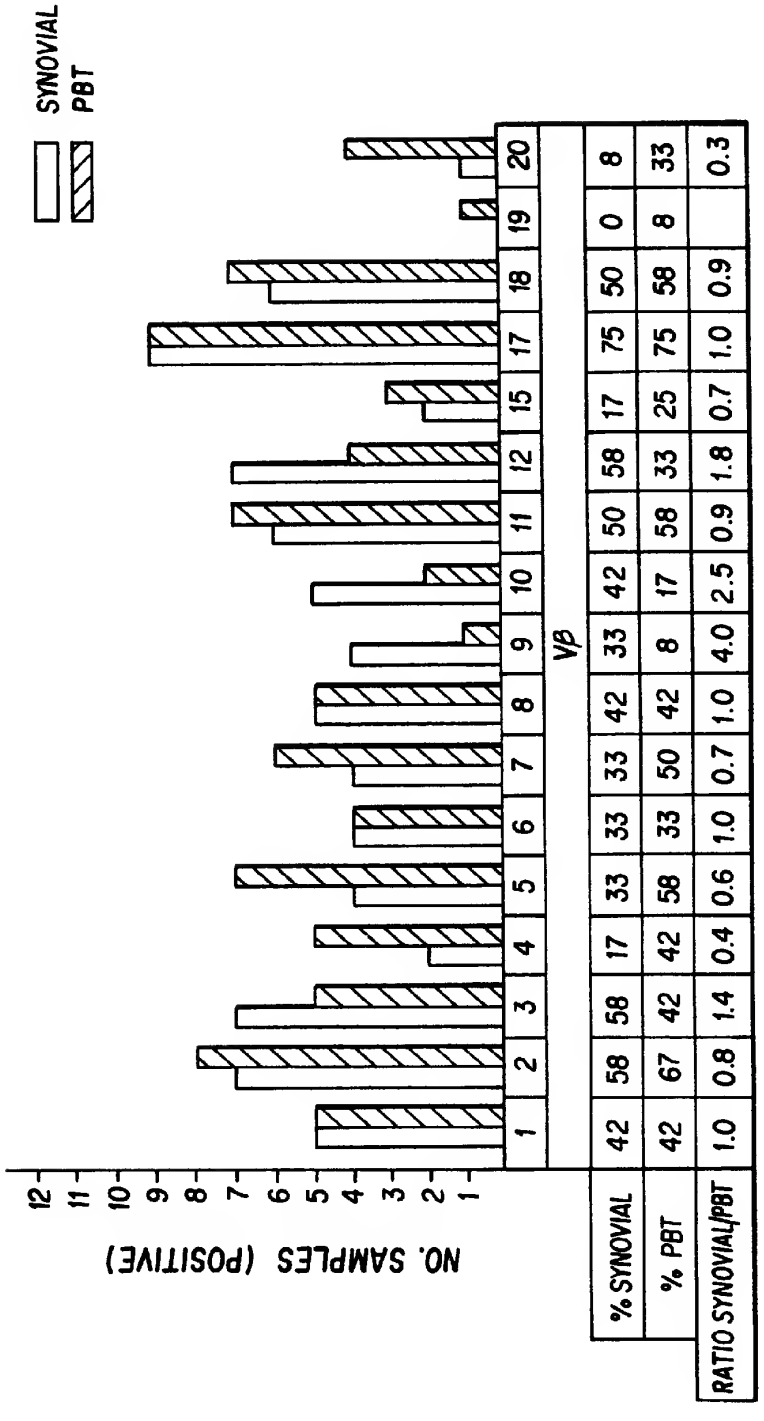
SYNOVIAL
PBT
NO. SAMPLES (POSITIVE)
12
11
10
9
8
7
6
5
4
3
2
1
Vβ
1 2 3 4 5 6 7 8 9 10 11 12 15 17 18 19 20
% SYNOVIAL 42 58 58 17 33 33 33 42 33 42 50 58 17 75 50 0 8
% PBT 42 67 42 42 58 33 50 42 8 17 58 33 25 75 58 8 33
RATIO SYNOVIAL/PBT 1.0 0.8 1.4 0.4 0.6 1.0 0.7 1.0 4.0 2.5 0.9 1.8 0.7 1.0 0.9 0.3

FIG. 19

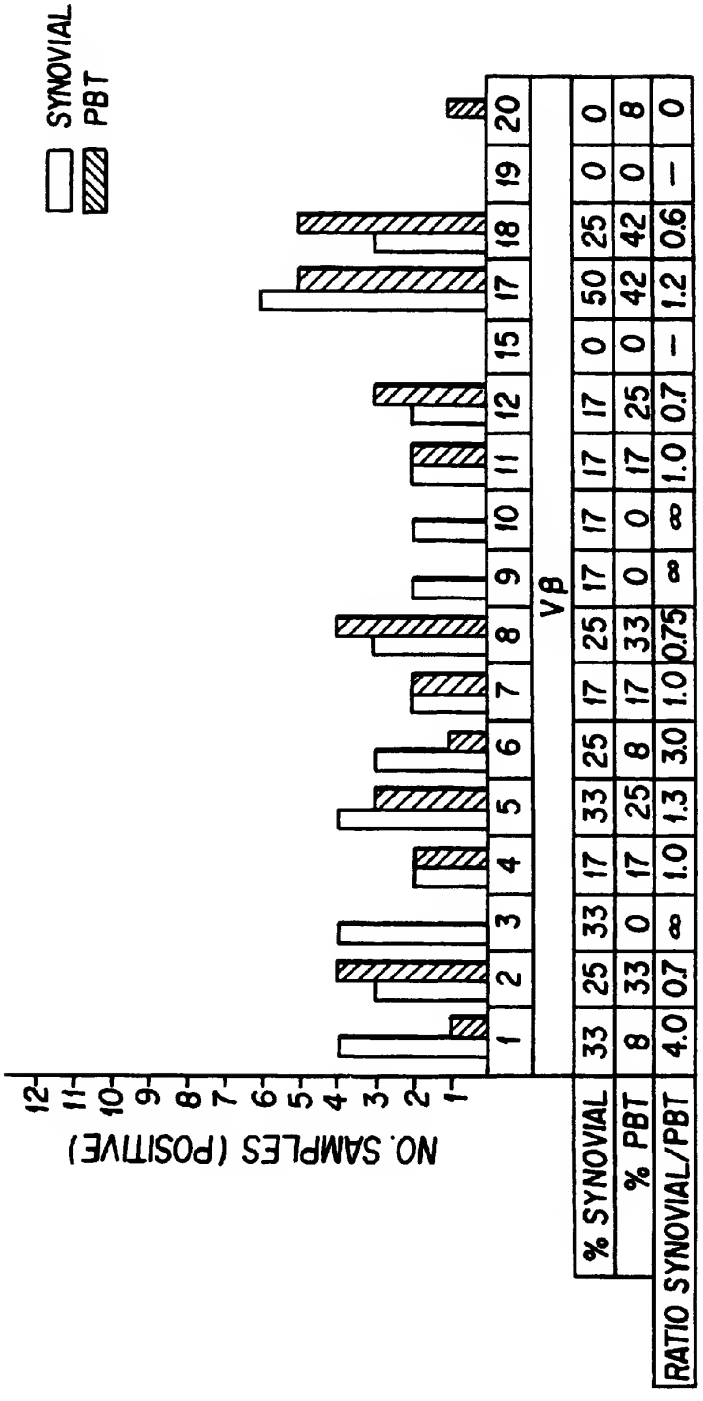
SYNOVIAL
PBT
NO. SAMPLES (POSITIVE)
12
11
10
9
8
7
6
5
4
3
2
1
Vβ
1 2 3 4 5 6 7 8 9 10 11 12 15 17 18 19 20
% SYNOVIAL 33 25 33 17 33 25 17 25 17 17 17 17 0 50 25 0 0
% PBT 8 33 0 17 25 8 17 33 0 0 17 25 0 42 42 0 8
RATIO SYNOVIAL/PBT 4.0 0.7 ∞ 1.0 1.3 3.0 1.0 0.75 ∞ ∞ 1.0 0.7 — 1.2 0.6 — 0

FIG. 20

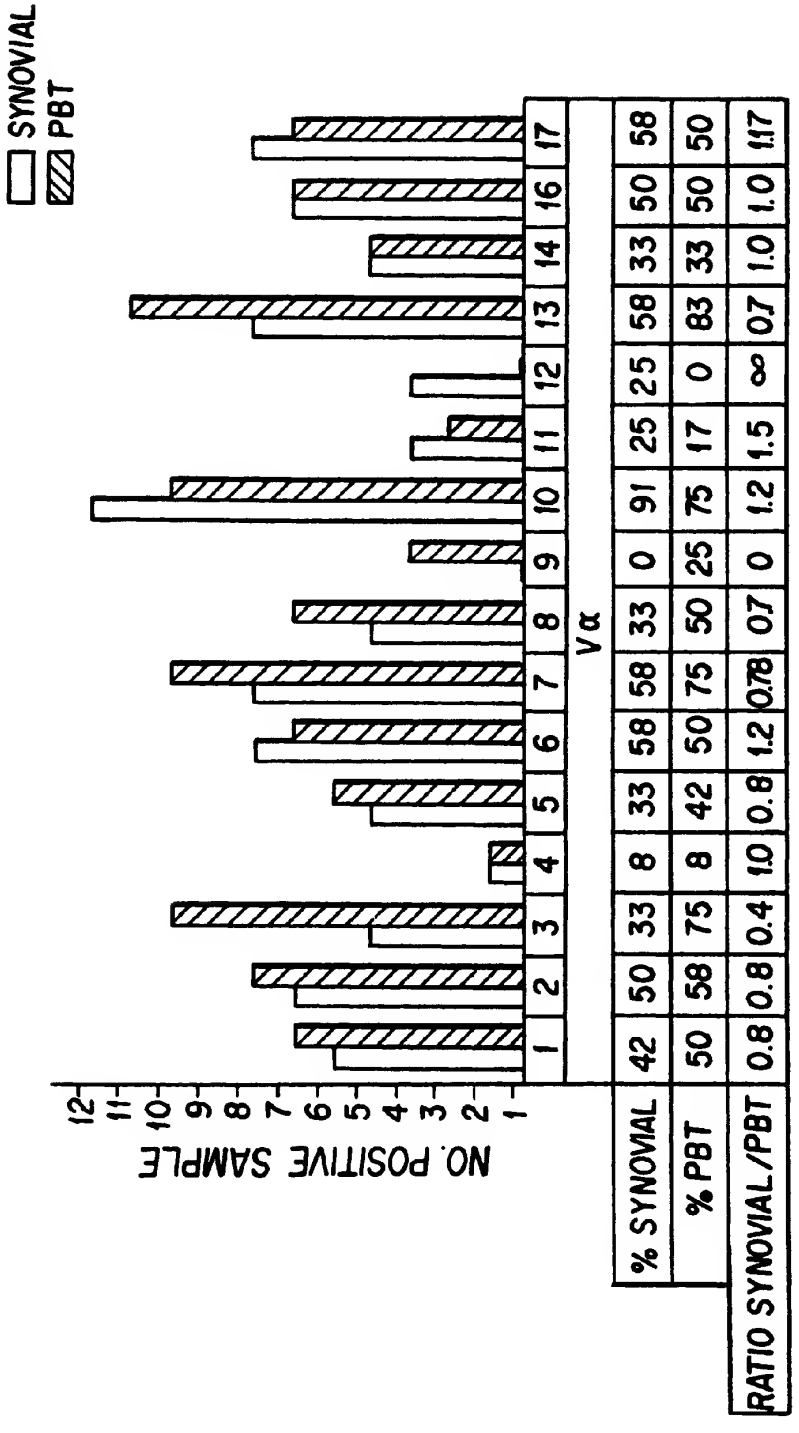
SYNOVIAL
PBT
NO. POSITIVE SAMPLE
12
11
10
9
8
7
6
5
4
3
2
1
Vα
1 2 3 4 5 6 7 8 9 10 11 12 13 14 16 17
% SYNOVIAL 42 50 33 8 33 58 58 33 0 91 25 25 58 33 50 58
% PBT 50 58 75 8 42 50 75 50 25 75 17 0 83 33 50 50
RATIO SYNOVIAL/PBT 0.8 0.8 0.4 1.0 0.8 1.2 0.78 0.7 0 1.2 1.5 ∞ 0.7 1.0 1.0 1.17

FIG. 21

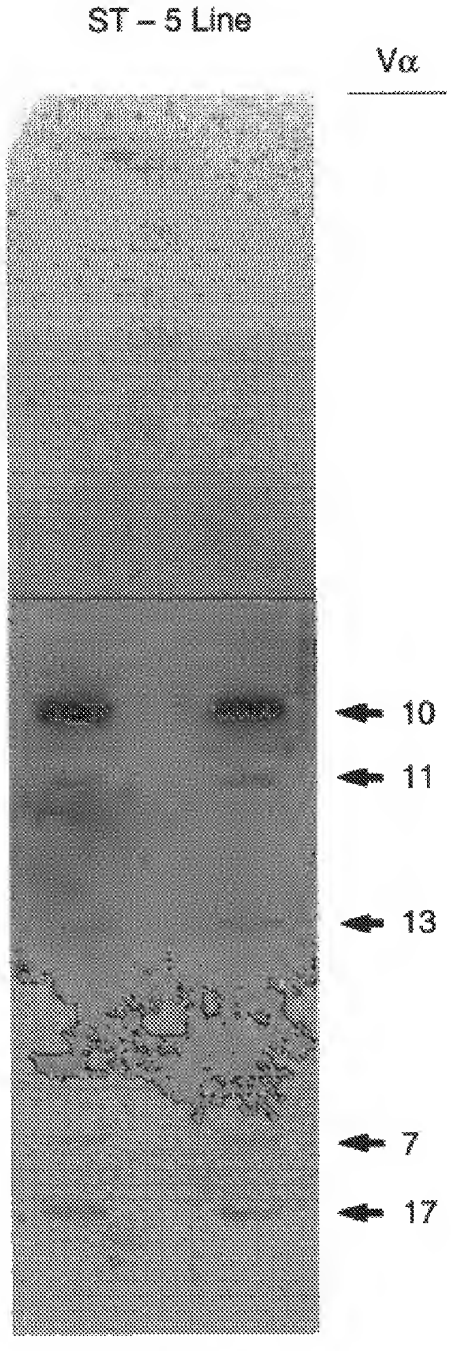


FIG. 22

# MONOCLONAL ANTIBODIES REACTIVE WITH DEFINED REGIONS OF THE T CELL ANTIGEN RECEPTOR

This is a division of U.S. application Ser. No. 08/083,408, filed Jun. 25, 1993, now U.S. Pat. No. 6,048,526, which is a division of U.S. application Ser. No. 07/449,692, filed Dec. 11, 1989 and now U.S. Pat. No. 5,223,426, which is a CIP of U.S. application Ser. No. 07/343,189, filed Apr. 25, 1989 and now abandoned, which is a CIP of U.S. application Ser. No. 07/284,511, filed Dec. 15, 1988 and now abandoned.

## 1. INTRODUCTION

The present invention is directed to monoclonal antibodies, which recognize defined regions of the T cell antigen receptor. The monoclonal antibodies of the invention have value in diagnosis and therapy and are useful tools for study of the immune system.

## 2. BACKGROUND OF THE INVENTION

### 2.1. THE T CELL ANTIGEN RECEPTOR

T lymphocytes interact with antigens through the T cell antigen receptor (TCR) complex. The TCR is a clone-specific heterodimer on T cells, which recognizes its target antigen in association with a major histocompatiblity antigen. The TCR has been shown to be noncovalently associated with the CD3 complex. TCR is highly polymorphic between T cells of different specificities. Approximately 90 percent of peripheral blood T cells express a TCR consisting of an α polypeptide and a β polypeptide. A small percentage of T cells have been shown to express a TCR consisting of a γ polypeptide and a δ polypeptide. (Regarding TCR molecules, see Davis and Bjorkman, 1988, Nature 334:395–402; Marrack and Kappler, 1986, Sci. Amer. 254: 36; Meuer et al., 1984, Ann. Rev. Immunol. 2:23–50; Brenner et al., 1986, Nature 322:145–159; Krangel et al., 1987, Science 237:1051–1055; Hata et al., 1987, Science 238:678–682; Hochstenbach et al., 1988, J. Exp. Med. 168:761–776). The chains of the T cell antigen receptor of a T cell clone are each composed of a unique combination of domains designatedvariable (V), [diversity (D),] joining (J), and constant (C) (Siu et al., 1984, Cell 37:393; Yanagi et al., 1985, Proc. Natl. Acad. Sci. USA 82:3430). Hypervariable regions have been identified (Patten et al., 1984, Nature 312:40; Becker et al., 1985, Nature 317:430). In each T cell clone, the combination of V, D and J domains of both the alpha and the beta chains or of both the delta and gamma chains participates in antigen recognition in a manner which is uniquely characteristic of that T cell clone and defines a unique binding site, also known as the idiotype of the T cell clone. In contrast, the C domain does not participate in antigen binding.

### 2.2. T CELL ANTIGEN RECEPTOR GENES

TCR genes, like immunoglobulin genes, consist of regions which rearrange during T cell ontogeny (Chien et al., 1984, Nature 312:31–35; Hedrick et al., 1984, Nature 308:149–153; Yanagi et al., 1984, Nature 308:145–149). In genomic DNA, each TCR gene has V, J, and C regions; TCR β and δ polypeptides also have D regions. The V (variable), D (diversity), J (junctional) and C (constant) regions are separated from one another by spacer regions in the DNA. There are usually many variable region segments and somewhat fewer diversity, junctional, and constant region segments. As a lymphocyte matures, these various segments are spliced together to create a continuous gene sequence consisting of one V, (D), J, and C region. TCR diversity, and



thereby T cell specificity, is derived from several sources (Barth et al., 1985, Nature 316:517–523; Fink et al., 1986, Nature 321:219–225): a multiplicity of germline gene segments (Chien et al., 1984, Nature 309:322–326; Malissen et al., 1984, Cell 37:1101–1110; Gascoigne et al., 1984, Nature 310:387–391; Kavaler et al., 1984, Nature 310: 421–423; Siu et al., 1984, Nature 311:344–349; Patten e al., 1984, Nature 312:40–46), combinatorial diversity through the assembly of different V, D, J, and C segments (Siu et al., 1984, Cell 37:393–401; Goverman et al., 1985, Cell 40:859–867), and junctional flexibility, N-region diversity and the use of either multiple D regions or any of the three translational reading frames for Dp segments. TCR diversity does not appear to arise from the somatic hypermutation mechanism observed for immunoglobulins (Barth et al., supra). As a result of these mechanisms, TCRs are generated which differ in their amino-terminal, or N-terminal, domains (called variable, or V regions, constructed from combinations of V, D, and J gene segments) but are the same elsewhere, including their carboxy-terminal, or C-terminal domains (called constant regions). Accordingly, an almost limitless repertoire of TCR is established.

The Vβ gene of the TCR appears to resemble most closely the immunoglobulin V gene in that it has three gene segments, Vβ, Dβ, and Jβ, which rearrange to form a contiguous Vβ gene (Siu et al., 1984, Cell 37:393–401). The β locus has been well characterized in mice, where it spans 700–800 kilobases of DNA and is comprised of two nearly identical C regions tandemly arranged with one D element and a cluster of 5–6 J elements 5' to each (Kronenberg et al., 1986, Ann. Rev. Immunol. 3:537–560). Approximately twenty to thirty Vβ regions are located upstream (5') to the D, J, and C elements (Behlke et al., 1985, Science 229:566–570) although Vβ genes may also be located 3' to the murine Cβ genes (Malissen et al., 1986, Nature 319:28). Study of the structure and diversity of the human TCR β-chain variable region genes has led to the grouping of genes into distinct Vβ subfamilies (Tillinghast et al., 1986, Science 233:879–883; Concannon et al., 1986, Proc. Natl. Acad. Sci. USA 83:6598–6602; Borst et al., 1987, J. Immunol. 139:1952–1959).

The γ-TCR gene was identified, first in mice (Saito et al., 1984, Nature 309:757–762; Kranz et al., 1985, Nature 313:762–755; Hayday et al., 1985, Cell 40:259–269) and then in humans (Lefranc et al., 1985, Nature 316:464–466; Murre et al., 1985, Nature 316:549–552). The human γTCR locus appears to consist of between five and ten variable, five joining, and two constant region genes (Dialynas et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:2619). The TCR α and δ locus are next to one another on human chromosome 14. Many TCR δ coding segments are located entirely within the a gene locus (Satyanarayana et al., 1988, Proc. Natl. Acad. Sci. USA 85:8166–8170 Chien et al., 1987, Nature 330:722–727; Elliot et al., 1988, Nature 331:627–631). It is estimated that there are a minimum of 45–50 Vα regions (Becker et al., Nature 317:430–434) whereas there are only approximately 10 Vδ regions (Chien et al., 1987, supra). In peripheral blood, two predominant Vδ genes appear to be expressed, namely, Vδ1 and Vδ2, identifiable by monoclonal antibodies, δTCS1 and BB3, respectively. Nucleic acid sequences of TCR α genes have been reported (Sim et al., 1984, Nature 312:771–775; Yanagi et al., 1985, Proc. Natl. Acad. Sci. USA 82:3430–3434; Berkout et al., 1988, Nucl. Acids Res. 16:5208).

### 2.3. ANTIBODIES TO THE T CELL ANTIGEN RECEPTOR

Clonotypic antibodies react only with a particular clone of T cells. Acuto et al. produced clonotypic monoclonal anti-

bodies against a human thymocyte cell line, and thereby identified the TCR in relatively undifferentiated $T3^+$ cells (1983, Cell 34:717–726). Meuer et al. showed that anti-TCR clonotypic monoclonal antibodies coupled to sepharose beads could induce production of interleukin-2(1984, Proc. Natl. Acad. Sci. 81:1509–1513). Anti-TCR clonotypic antibody directed toward the CT8 cell line could only block cytotoxic effector cell function of that T cell line (Meuer et al., 1984, Ann. Rev. Immunol. 2:23–50). Antibodies which recognize TCR from many T cell lines recognize shared epitopes, or framework regions, of TCR peptides. Brenner et al. found that different cloned T cell lines shared antigenic determinants, none of which appeared to be accessible at the cell surface (1984, J. Exp. Med. 160:541–551). β-Framework-1 (βF1) monoclonal antibody reacts with a "hidden determinant" on the surface of viable T cells, and recognizes the TCR β polypeptide in Western blots (Brenner et al., 1987, J. Immunol. 138:1502–1509). Another framework antibody, WT31, originally thought to be a framework reagent is useful in cell binding, but is inefficient in immunoprecipitation studies (Spits et al., 1985, J. Immunol. 135:1922–1928). WT31 now appears to recognize a CD3 determinant.

2.4. RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA), a chronic, recurrent, inflammatory disease primarily involving joints, affects 1–3% of North Americans, with a female to male ratio of 3:1. Severe RA patients tend to exhibit extra-articular manifestations including vasculitis, muscle atrophy, subcutaneous nodules, lymphadenopathy, splenomegaly and leukopenia. Spontaneous remission may occur; other patients have brief episodes of acute arthritis with longer periods of low-grade activity; still others progress to severe deformity of joints. In some patients with rheumatoid arthritis, particularly those with long-standing disease, a constellation of symptoms called "Felty's syndrome" develops, in which the typical arthropathy is accompanied by splenomegaly and neutropenia. It is estimated that about 15% of RA patients (severe RA and Felty's syndrome) become completely incapacitated ("Primer on the Rheumatic Diseases, 8th edition, 1983, Rodman, G. P. & Schumacher, H. R., Eds., Zvaifler, N. J., Assoc. Ed., Arthritis Foundations, Atlanta, Ga.

The antigenic stimulus initiating the immune response and consequent inflammation is unknown. Certain HLA types (DR4, Dw4, Dw14 and DR1) have an increased prevalence of RA, perhaps leading to a genetic susceptibility to an unidentified factor which initiates the disease process. The association with DR4 is highest for Felty's Disease and severe RA (Westedt, M. L., et al., Annals of Rheumatic Diseases, 1986, 45, 534–538). Relationships between Epstein Barr virus and RA have been suggested. Synovial lymphocytes produce IgG that is recognized as foreign and stimulates a local immune response with production of anti-IgG-antibodies (rheumatoid factors). Immune complexes are formed by activation of the complement system which results in inflammation including activation of lysozyme and other enzymes. Helper T cell infiltration of the synovium and liberation of lymphokines such as IL6 lead to further accumulation of macrophages and slowly progressing joint destruction (erosions).

The approach to drug treatment in rheumatoid arthritis has been described as a pyramid ("primer on the Rheumatic Diseases", supra). First line agents include aspirin and NSAIDS (non-steroidal anti-inflammatory drugs). When these agents fail, gold salts, penicillamine, methotrexate, or antimalarials, known as conventional second line drugs, are considered. Finally, steroids or cytotox are tried in patients with serious active disease that is refractory to first and second line treatment. Cyclosporine is now suggested to have a role in the treatment of patients whose disease is unresponsive to aspirin, NSAIDS, gold or penicillamine. However, the current experimental drugs to treat severe RA patients may prove too toxic even if they are effective.

Numerous efforts have been directed to developing safer and more efficacious immunotherapy to replace these toxic drugs. Severe RA patients who were treated with total lymphoid irradiation or thoraic duct drainage experienced significant improvement of disease symptoms. These procedures are not suitable for routine application. Due to these encouraging findings, however, and to the demonstration of the presence of T cells in the synovial infiltrate, it is possible to design new immunotherapies to specifically eliminate T cells. Most of these new experimental immunotherapies are targeted toward all or the bulk of T cells, and thus may produce significant side effects. A better approach for selective immunotherapy may be to eliminate only the small proportion of T cells that are involved in RA.

2.5. ROLE OF T CELLS IN RHEUMATOID ARTHRITIS

Evidence has accumulated supporting a role for T-cells in the pathogenesis of rheumatoid arthritis (RA). The synovial tissue and surrounding synovial fluid of patients with rheumatoid arthritis (RA) are infiltrated with large numbers of cells. Activated and resting T cells can mediate tissue damage by a variety of mechanisms including the direct cytotoxicity of target cells expressing specific antigen in combination with the appropriate HLA restricting elements. The strong association of certain HLA products with RA has led researchers to implicate T cells in the autoimmune destruction of RA patient joints. In fact, HLA DR4, Dw4 and Dw14 gene products are among the major class II molecules that contribute significantly to disease susceptibility in RA patients (Nepom, B., et al., 1987, Abstracts of Amer. Rheumatism Assoc., p. S25; Todd, J. A., et al., 1988, Science 240:1003–1009), and they are capable of restricting antigen recognition of CD4+ T cells, primarily. Other autoimmune diseases also show a high correlation between disease susceptibility and HLA expression (Table 1).

This genetic basis of disease risk has resulted in phenotypic analysis of the T cells found within diseased joints. Previously, comparisons of T cells from RA joints and RA peripheral blood (PB) demonstrated significant differences in CD4 or CD8 phenotype, therefore implying a selection of T cells involved in disease activity. Most studies agree that synovial tissue-infiltrated T cells were mostly CD4+ helper-inducer (4B4+) cells (Duke, O., et al., 1987, Arth. Rheum., 30, 849) while the PB usually contained a mixture of CD4+ and CD8+ cells including both helper-inducer cells and suppressor-inducer cells (2H4+) (Emery, P., et al., 1987, Arth. Rheum., 30, 849). In contrast, there is additional evidence that the CD4+ infiltrate may be predominantly suppressor-inducer cells (2H4+) (Mikasaka, N., et al., 1987, Amer. Rheum. Abstracts, p. S39).

2.6. γδ POSITIVE T CELLS

γδ TCR may be the principal TCR in selected sites such as the skin or other organs. Although the function of the γδ positive T cells is largely unknown, they appear to be involved in non-MHC-restricted cytotoxicity and IFN-γ production. $γδ^+$ T cells are known to secrete a variety of lymphokines, such as TNF alpha, IL2 and IL4 (Bluestone, J. A. and Matis, L. A., 1989, J. Immunol. 142, 1785–1788). The total population of $δ^+$ T cells can be identified by the monoclonal antibody, TCRδ1, which recognizes a major framework determinant on the δ TCR (Band, H., et al., 1987, Science, 238, 682). A subset of γδ positive T-lymphocytes

can be identified by the monoclonal antibodies, δTCS1 (anti-$V_δ1$; Wu, Y-J., et al., 1988, J. Immunol. 141, 1476–1479) and BB3 (anti-$V_δ$ TCR, Bottino, C., et al., 1988, J. Exp. Med., —, 491–505). A study by Grossi, C. E., et al. (Proc. Natl. Acad. Sci., 1989) indicated that δTCS1$^+$ T cells exhibit motile cell morphology and migrate in tissue culture. δTCS1$^+$ T cells were also shown to be potent killer T cells (Rivas, A., et al., 1989, J. Immunol., 142, 1840–1846).

## 3. SUMMARY OF THE INVENTION

The present invention is directed to monoclonal antibodies which recognize defined regions of the T cell antigen receptor (TCR). The antibodies of the invention bind to epitopes of the variable, diversity, joining, and/or constant regions of the alpha, beta, gamma, or delta chains of the T cell antigen receptor.

In a specific embodiment, the invention provides monoclonal antibodies which are reactive with a constant region of the alpha chain of the TCR. In particular embodiments, the invention relates to the two monoclonal antibodies, termed αF1 and αF2, which react with two different epitopes on the framework, or constant, region of the α monomer of the TCR molecule. In various embodiments of the invention, αF1 or αF2, or both, or fragments or derivatives thereof, can be used to bind to the α TCR constant region amino acid sequences, either as part of an intact TCR complex or α peptide, or a frant thereof.

In another specific embodiment, the invention is directed to monoclonal antibodies reactive with a variable region of the beta chain of the TCR. In a preferred embodiment of the invention, the monoclonal antibodies react with a "minor framework" region of the TCR beta chain, and thereby recognize a subpopulation of T cells. In particular, the invention provides two monoclonal antibodies, termed W112 and 2D1, which react with β-chain variable regions Vβ5.3 and Vβ8.1, respectively, and thereby recognize between 0.3 to 5% and 0.5 to 13% of peripheral blood lymphocytes, respectively. In various embodiments of the invention, W112 or 2D1, or fragments or derivatives thereof, can be used to bind with βTCR variable region amino acid sequences, either as part of an intact TCR or peptide, or T cell-surface molecule, or a fragment thereof.

In another specific embodiment, the invention is directed to monoclonal antibodies reactive with a variable region of the delta chain of the TCR. In a preferred embodiment of the invention, the monoclonal antibodies react with the Vδ1 region of the TCR delta chain, and thereby recognize a subpopulation of T cells.

In a further specific embodiment, the invention is directed to a particular monoclonal antibody, δTCS1, which is of the IgG2a isotype.

The monoclonal antibodies of the invention have value in the diagnosis and therapy of conditions and diseases affecting the immune system.

In particular embodiments of the invention, rheumatoid arthritis or Felty's syndrome may be diagnosed by detecting increased percentages of total T cells which express certain delta or beta chain T cell receptor variable region genes in a patient sample. In specific embodiments of the invention, rheumatoid arthritis may be diagnosed by dtecting increased percentages of total T cells which express Vδ1, Vβ3, Vβ9, or Vβ10 T cell receptor variable regions in a patient sample. In a preferred embodiment of the invention, rheumatoid arthritis may be diagnosed by detecting increased percentages of total T cells which are δTCS1 positive in a patient sample.

In further particular embodiments of the invention, rheumatoid arthritis may be treated by administering a therapeutically effective amount of a monoclonal antibody, or fragment or derivative thereof, which recognizes an epitope of the variable region of the beta chain or the delta chain of a T cell antigen receptor. According to specific embodiments, monoclonal antibodies which recognize epitopes of Vδ1, Vβ3, Vβ9, or Vβ10 variable regions of the T cell antigen receptor may be used to treat rheumatoid arthritis.

The invention also provides for therapeutic compositions comprising the monoclonal antibodies of the invention.

### 3.1. ABBREVIATIONS AND DEFINITIONS

As used herein, the following terms will have the meanings indicated:

C=constant

D=diversity

ELISA=enzyme linked immunosorbent assay

J=joining

mAb=monoclonal antibody

PBL=peripheral blood lymphocytes

PMA=phorbol 12-myristate 13-acetate

SDS-PAGE=sodium dodecylsulfate polyacrylamide gel electrophoresis

TCR=T cell antigen receptor

V=variable

anti-clonotypic antibody=an antibody that reacts solely with the T cell clone against which it was raised. Also referred to as an anti-idiotypic antibody.

anti-minor framework antibody=an antibody that reacts with a minor framework determinant present on a subset of T cells. Anti-minor framework antibodies recognize small percentages of PBLs, i.e., less than 20% in a normal subject. Anti-minor framework antibodies can be used to define closely related TCRs or TCR families.

anti-major framework antibody=an antibody that reacts with a major framework determinant present on a large population of T cells. Anti-major framework antibodies will recognize at least 20% of PBLs in a normal subject.

RES=reticuloendothelial system.

RA=Rheumatoid Arthritis

PB=peripheral blood

ST-line=RA synovial tissue-derived T cells

PB-T=peripheral blood-derived T cells

FS=Felty's Syndrome

SSA=Seronegative Spondyloarthropathies

EBV=Epstein-Barr virus

PBS=phosphate buffered saline

NK=natural killer

NST-line=non-RA synovial tissue-derived T cells

HLA=human leukocyte antigen

## 4. DESCRIPTION OF THE FIGURES

FIG. 1. Immunoprecipitation of $^{125}$I labeled HPB-cell lysates and Molt-13 cell lysates. HPB- and Molt-13 cells were surface labeled with $^{125}$I and lysed in 1% NP-40. Cell lysates were incubated with mAb and the precipitated immune complex was applied to 10% SDS-PAGE. Electrophoresis was run under non-reducing (A) and reducing conditions (B). The precipitated TCR proteins were detected by autoradiography.

FIG. **2**. TCR immunoprecipitation from Jurkat cells and PBL with αF1 and αF2. $^{125}$I labeled Jurkat cells and PBL were lysed in 1% NP-40 and incubated with αF1 and αF2 and control mAb. The immune complex precipitated was revealed by 10% SDS-PAGE under non-reducing conditions.

βF1 antibody was a positive control for immunoprecipitating the αβTCR. δTCS1 antibody reacts with $\delta\gamma^+$ cells, not ab cells.

FIG. **3**. Immunoperoxidase staining of human thymus and tonsilar tissue. A and B are thymus cortex; C and D are thymus medulla; E and F are human tonsil. βF1 stains about 70% of cells in cortex (A), over 90% in medulla (C) and all T cells in the interfollicular region of tonsil (E). A similar staining pattern is observed when αF1 is used (B, D, and F). Note that αF1 stains less thymus cortex cells (B).

FIG. **4**. Competition assay of TCRα peptides with HPB-TCR. Two-fold dilutions of free α141–159 (amino acids 141–159 of TCRα)(open circle) or α212–231 (amino acids 212–231 of TCRα)(solid circle) were mixed with 10% HPB-lysates and incubated with αF1 (a) or αF2 (b) in a TCR-ELISA assay. Peptide concentration is shown on the X-axis in logarithmic scale. αF1 and αF2 were used at 4 μg/ml.

FIG. **5**. Immunoprecipitation of in vitro translated α chain T cell antigen receptor protein witb mAb αF1 and αF2. Aliquots of protein mixture containing both α chain and rabbit globin (lane 6) were immunoprecipitated separately with anti-Cα antibodies, αF1 (lane 1) and αF2 (lane 2), and isotype matched, irrelevant antibodies in lane 3 and lane 4. Lane 5, in vitro translation in the absence of any exogenous RNA. Samples were analyzed on a 12.5% SDS-PAGE which was dried and subsequently autoradiographed.

FIG. **6**. A. A schematic of the hybridoma screening ELISA. Microtiter plate wells were coated with goat anti-mouse Ig Fc and washed to remove unbound reagent. Non-specific sites were blocked with bovine serum albumin. Aliquots of hybridoma supernatants were added to the wells with blocking buffer, incubated, and followed by washing to remove unbound proteins. NP40 generated cell lysates from αβ TCR cell lines were added, wells were washed, and biotin-conjugated βF1 $F(ab)_2$ was added. After washing to remove unbound reagent, HRP conjugated streptavidin was added, wells were washed, and color was developed using an HRP substrate, o-phenylenediamine.

B. Comodulation assay: Jurkat cells were incubated with antibodies overnight, washed, incubated with FITC-labeled OKT3 and analyzed by flow cytometry. Antibody 2D1 caused a dramatic reduction (over 90%) of OKT3 surface staining. C305, an anti-Jurkat IgM isotype antibody and OKT3 also modulated the CD3 expression, while isotype-matched control antibody W4 did not. This data indicated that the epitope recognized by 2D1 is on the CD3-TCR complex.

FIG. **7**. A. Immunoprecipitation of HPB with W112 and control antibodies under non-reducing conditions followed by SDS-PAGE under reducing conditions. W112 and βF1 precipitated the αβ TCR heterodimer of 48 kD (α) and 40 kD (β). Normal mouse serum did not precipitate the αβ TCR heterodimer.

B. Immunoprecipitation of Jurkat cell lysates with 2D1 and control antibodies under non-reducing conditions followed by SDS-PAGE under reducing conditions. 2D1 precipitated the α,β TCR heterodimer of 48 kD (α) and 40 kD (β). βF1 and αF1 precipitated the same 48 kD and 40 kD bands, while negative control antibody δTCS1 did not.

FIG. **8**. Western blot analysis using HPB cell membranes. W112 recognized the 40 kD TCR β chain protein as indicated by the arrow. βF1 also detected the 40 kD band. Normal mouse serum did not react with this band. αF1 detected a different band of a higher molecular weight.

FIG. **9**. Immunoprecipitation of metabolically labelled Jurkat cell lysates. 2D1 precipitated the 40 kD β chain TCR, as did βF1. Isotype-matched negative control antibody LL-112 was unreactive.

FIG. **10**. Flow cytometric analysis of peripheral blood lymphocytes (PBLs) from one normal donor using W112 and 2D1 antibodies. The positive control pan-T monoclonal antibody OKT3 stained 70% of the PBLs. W112 stained a small subpopulation consisting of 3% of the PBLs and 2D1 stained a small subpopulation of 5.5% PBLs. Normal mouse serum did not react with any of the PBLs.

FIG. **11**. Levels of TCRδ1 bearing T cells (left panel) and δTCS1 bearing T cell (right panel) in the PB of patients with RA, FS and NML. Arithmetic mean values are represented by the 'X' and the error bars represent ±1 standard error (S.E.).

FIG. **12**. Ratio of δTCS1/TCRδ1 bearing T cells in patients with RA, FS and NML. Notations as per FIG. **11**.

FIG. **13**. Relationship between the percent of TCRδ1 T cells and the percent of δTCS1 T cells in patients with FS.

FIG. **14**. Levels of TCRδ bearing T cells (upper panel) and δTCS1 bearing T cells (lower panel) in the PB and SF of RA and SSA patients. Notations as per FIG. **11**.

FIG. **15**. Ratio of δTCS1/TCRδ1 bearing T cells in the PB and SF of RA patients and SF of patients with SSA. Notations as per FIG. **11**.

FIG. **16**. Synovial derived T cell (ST-13) cytotoxicity of type A synovial target cells and the cold target inhibition by K562. Effector to $^{51}$Cr labelled target cell ratio was 1:1 while increasing concentrations of unlabelled target cells were added. The standard deviations did not exceed 4%. The spontaneous 51Cr release for ST-13 type A synoviocytes was 38%.

FIG. **17**. Assessment of patient benefit. There are several standard criteria to assess whether arthritis patients are benefiting from treatment. At each visit, patients are scored according to the ten criteria listed and an overall additive score is determined. This score is used to establish the effectiveness of the drug treatment. Other parameters can also be measured. These include blood counts, liver function tests, Westegren sedimentation rates, rheumatoid factor tests, etc.

FIG. **18**. Analysis of Vβ Gene Usage in Synovial Tissue Derived T Cell Line. Line ST-2, derived from the synovial membrane infiltrating cells of a rheumatoid arthritis patient, was analyzed for TCR Vβ expression using the cDNA, PCR amplification, slot blot hybridization protocol. The left part of the figure represents the autoradiograph obtained when the panel of Vβ genes was hybridized with the ST-2 amplified TCR specific cDNA probe. The right part of the figure is the densitometry trace of the autoradiograph.

FIG. **19**. Detection of Vβ Gene Usage in Rheumatoid Arthritis T Cells. This figure is a tabulation of the results of the expression of the panel of Vβ genes in 12 paired synovium tissue derived and peripheral blood derived T cell lines from rheumatoid arthritis patients. For the top part of the figure, the vertical axis represents the number of samples that were positive for a particular Vβ. The individual Vβ genes are indicated on the horizontal axis. Data derived from synovial T cells and peripheral blood T cells are plotted in

pairs as the open and crosshatched bars, respectively. For the bottom part of the figure, the frequencies of the individual Vβ genes in the 12 patient samples are shown (%synovial and %PBT). To indicate preferential usage of Vβ genes the ratio of occurrence in the synovial/peripheral blood samples is shown.

FIG. **20**. Detection of Dominant Vβ Gene Usage in Rheumatoid Arthritis T Cells. This figure is similar to FIG. **19**, except that the tabulated data includes only the expression of the most frequently occurring Vβ genes as determined by the densitometry trace. The most frequent or dominant Vβ was determined from the highest peak height which was used as a standard. Any Vβ gene with a corresponding densitometry peak with height greater than 50% of the standard was used in the tabulation.

FIG. **21**. Detection of Vα Gene Usage in Rheumatoid Arthritis T Cells. This figure is similar to FIG. **19**, except the patient samples were analyzed for Vα gene expression. The data in this figure represents the total Vα expression observed, not the dominant or most frequently expressed Vα. 85% of the T cell samples tested expressed Vα10 at a densitometry peak height 100-fold greater than for the other Vβ genes.

FIG. **22**. Vβ Gene Usage in the Synopy Tissue Derived T Cell Line ST-5. The dominantly expressed Vβ gene is Vα10, although other Vβ genes are expressed as minor populations.

## 5. DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to monoclonal antibodies which recognize defined regions of T cell antigen receptor (TCR) chains. Such antibodies bind to epitopes of the variable, diversity, joining, and/or constant regions of the alpha, beta, gamma, or delta chains of the T cell antigen receptor. As used herein, a monoclonal antibody reactive with the "variable region" of the TCR shall be construed to be a monoclonal antibody reactive with an epitope of the V region or a combination epitope of the V region or a combination epitope of the V-D or V-D-J regions; a monoclonal antibody reactive with a "variable region" of TCR may recognize an idiotype, a clonotype, or, preferably, may recognize a minor framework region expressed by a subgroup of T lymphocytes. The term minor framework region refers to a region of the TCR which is not shared by all TCR molecules, but is also not unique to a particular clone of T cells. In a specific embodiment, the monoclonal antibodies of the invention are reactive with a constant region of the alpha chain of the T cell antigen receptor. In another embodiment, the monoclonal antibodies are reactive with a variable region of the beta chain of the T cell antigen receptor. In particular, such an anti-TCRβ mAb can recognize Vβ5.3. In another particular embodiment, such an anti-TCRβ mAb can recognize Vβ8.1. In another embodiment, the monoclonal antibodies are reactive with a variable region of the delta chain of the T cell antigen receptor. In particular such an anti-TCRδ mAb can rsgnize Vδ1.

The monoclonal antibodies of the invention, and fragments, derivatives, and analogues thereof, have uses in diagnosis and therapy.

In specific embodiments of the invention, monoclonal antibodies which bind to Vδ1, Vβ3, Vβ9, or Vβ10 variable regions of the T cell antigen receptor may be utilized in the diagnosis and therapy of rheumatoid arthritis.

### 5.1. GENERATION OF MONOCLONAL ANTIBODIES REACTIVE WITH DEFINED REGIONS OF THE T CELL ANTIGEN RECEPTOR

The monoclonal antibodies of the invention are directed to an epitope of a defined region of the T cell antigen receptor. A monoclonal antibody to an epitope of the T cell can be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein (1975, Nature 256:495–497), and the more recent human B cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77–96), and trioma techniques.

In one embodiment, the monoclonal antibodies may be of human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies. Human monoclonal antibodies may be made by any of numerous techniques known in the art (e.g., Teng et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:7308–7312; Kozbor et al., 1983, Immunology Today 4:7279; Olsson et al., 1982, Meth. Enzymol. 92:3–16). Chimeric antibody molecules may be prepared containing a mouse (or rat, or other species) antigen-binding domain with human constant regions (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851; Takeda et al., 1985, Nature 314:452). For therapeutic purposes, antibodies can be further humanized, by producng human constant region chimeras, where the conserved or framework regions of the antigen-binding domain are also from human sequences, and only the hypervariable regions are non-human.

As part of the production of the monoclonal antibodies of the invention, various host animals, including but not limited to rabbits, mice, and rats can be immunized by injection with purified T cell antigen receptors or polypeptides or fragments thereof, a recombinant or synthetic version thereof, or T lymphocytes.

Various cell lines can be used as immunogens to generate monoclonal antibodies to the V region of human T cell antigen receptors, including, but not limited to, those cell lines listed in Table 4 (Toyonaga et al., 1987, Ann. Rev. Immunol. 5: 585–620). Any T cell line could be used, including any γδ+ cell line, such as (but not limited to) PEER, MOLT 13, WM 14, AK119, etc., provided that TCR is expressed on the cell surface, as in the MOLT 13 cell line. Note that antibodies to known V, D, J, DJ, VJ, VDJ or combinations thereof can also be generated by immunizing with these cell lines. V, D, J, and C region α, β, γ or δ expression can be determined in any cell line by well known procedures including cDNA sequencing, in situ hybridization, polymerase chain reaction analysis, Northern analysis, Southern analysis, immunoassay, or flow cytometry, to name but a few.

Whole cells that can be used as immunogens to produce TCR specific antibodies also include recombinant transfectants. For example, $\beta^-$ Jurkat cells can be reconstituted by transfection with an β cDNA to produce intact αβ TCR on the cell surface (Ohashi, P. S., et al., Nature, 316:606–609). These transfectant cells could then be used as immunogen to produce a or β TCR antibodies of preselected specificity. Other examples of such transfectant cells have been reported (Kaye, J. and Hedrick, S. H., 1988, Nature 336:580–583; Dembic, Z., et al., 1986, Nature 320: 232–238; Saito, T., et al., 1987, Nature 325:125–130), but any procedure that works to express transfected TCR genes on the cell surface could be used to produce the whole cell immunogen. This includes, but is not limited to, eukaryotic expression systems

utilizing a phospholipid anchor domain (International Patent Application no. PCT/US88/02648 published Feb. 9, 1989). Screening procedures that can be used to screen hybridoma cells expressing different anti-TCR antibodies include but are not limited to (1) enzyme linked immunosorbent assays (ELISA), (2) flow cytometry (FLOW) analysis, (3) immunoprecipitation, and (4) the ability to comodulate the CD3 antigen (part of the TCR-CD3 complex present on the surface of T cells) off of the surface of cells. The comodulation and FLOW screening procedures are preferred for the selection of antibodies potentially useful in therapy since these procedures will result in selection of antibodies that are able to recognize intact TCR on live cells due to the inherent properties of these techniques.

Many different formats of an ELISA that can be used to screen for anti-TCR antibodies can be envisioned by one skilled in the art. These include, but are not limited to, formats comprising purified, synthesized or recombinantly expressed TCR polypeptide attached to the solid phase or bound by antibodies attached to the solid phase or formats comprising the use of whole T cells or T cell lysate membrane preparations either attached toMhe solid phase or bound by antibodies attached to the solid phase. Samples of hybridoma supernatants would be reacted with either of these two formats, followed by incubation with, for instance, a goat anti-mouse immunoglobulin complexed to an enzyme-substrate that can be visually identified.

In another screening method, supernatants of antibody producing hybridomas can be screened by FLOW in a number of different ways as is known by one skilled in the art. One screening procedure involves the binding of potential antibodies to a panel of T cells that express well-known TCRs on their surface. Generally, antibodies that react with intact TCR are detected by this analysis, but the T cells can be fixed slightly with e.g. ethanol, in which case antibodies reacting with denatured TCR polypeptide can be identified. FLOW assays can also be formatted where potential antibodies are screened by their ability to compete with the binding of a known antibody for the TCR present on a T cell.

It is also possible to screen antibodies by their ability to immunoprecipitate a known TCR as analyzed by SDS-polyacrylamide gel electrophoresis or Western blot analysis. An advantage to this assay is that it is possible to identify the chain of the TCR heterodimer that the anti-TCR antibodies are recognizing.

Additionally, screening may be carried out by observing the comodulation of the CD3 antigen. The TCR normally exists on the surface of T cells as a complex with the chains of the CD3 complex. When an antibody binds to this TCR:CD3 complex, the complex in turn becomes internalized in the T cell and disappears from the cell surface. Thus, if T cells are reacted with an antibody specific to the TCR and the complex becomes internalized, a further reaction with an anti-CD3 specific antibody will result inubstantially no detection of CD3 bearing cells by FLOW analysis. This comodulation screening procedure detects antibodies that are able to interact with intact TCR on the surface of T cells.

Many additional screening assays, such as those based upon competition with anti-TCR antibodies of known specificity or the ability to cause T cells expressing known TCRs to proliferate in culture, will be known to those skilled in the art and can be used for the selection of appropriate antibodies.

A molecular clone containing a DNA sequence of an antibody to an epitope of a T cell surface molecule can be prepared by known techniques. Recombinant DNA methodology (see e.g., Maniatis et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) may be used to construct nucleic acid sequences which encode a monoclonal antibody molecule, or antigen binding region thereof.

Antibody molecules may be purified by known techniques, e.g., immunoabsorption or immunoaffinity chromatography, chromatographic methods such as HPLC (high performance liquid chromatography), or a combination thereof, etc.

Once antibodies of the desired specificity are generated, other antibodies of the same epitope specificity can be selected. Antibodies of such similar epitope specificity can be selected, for example, by observing the ability of such antibody or binding region thereof to inhibit the binding of the antibody of known specificity to its antigen. Various competitive binding assays known in the art can be used.

## 5.2. FRAGMENTS AND DERIVATIVES OF HONOCLONAL ANTI-BODIES REACTIVE WITH DEFINED REGIONS OF THE T CELL ANTIGEN RECEPTOR

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

Various chemical derivatives of the antibodies of the invention can also be produced. For example, immunoconjugates consisting of an antibody molecule or binding region thereof bound to a label such as a radioisotope or other tracer molecule can be made by techniques known in the art. Alternatively, the antibody molecule or fragment thereof can be bound to a therapeutically useful molecule which is targeted to its desired site of action by virtue of the antibody's binding specificity. As one example of such an embodiment, a cytotoxic compound can be conjugated to an antibody of the invention which is specific to a region of the TCR on lymphocytes which are the causative agents of an autoimmune disorder. The cytotoxic compound in conjugate form is thus targeted to the implicated T lymphocytes.

In addition, isotypes of the antibodies of the invention can be switched in order to optimize clinical applications. For example, some isotypes (such as IgG2a) are superior effectors of antibody dependent cell cytotoxicity reactions. Likewise, some isotypes (such as IgG2a) are more readily eliminated from the circulation via the Fc receptors present on cells of the reticuloendothelial system and sequestered in the spleen than others, and, if bound to a target cell (which, for example, may be an effector of autoimmune disease), would be more efficient at removing the target cell from sites of active disease. Accordingly, certain antibody isotypes may be preferable to others, depending on the intended clinical application. Therefore, hybridomas are screened for TCR specific mAbs using a TCR ELISA designed to select for desirable isotypes, such as IgG. In addition, a different isotype may be generated by spontaneous isotype switching and directed toward various uses. A method exists which facilitates selecting for the isotype of interest; the procedure for isotype switching of IgGl to IgG2a presented as an example, is as follows: Hybridoma cells are grown in log phase for a 2–3 week period prior to a magnetic bead negative selection. For the magnetic bead selection, super paramagnetic iron oxide particles coated with a goat anti-mouse antibody preparation including all IgG isotype classes (Biomag® beads purchased from Advanced

Magnetics, Inc.) may be used. For the process of switching an antibody isotype from IgG1 to IgG2a, it is necessary to block the IgG2a binding sites on the antibody coated beads by incubating with an irrelevant antibody having an IgG2a isotype. $10^8$ hybridoma cells are then incubated with the beads, allowing cells expressing IgG1, IgG2b and IgG3 isotypes to bind to the beads and be removed magnetically from the population. This negative selection should be repeated several times. The remaining cell population, depleted of IgG1, IgG2b and IgG3 bearing cells and enriched for IgG2a bearing cells is plated in 15 microtiter plates at a cell density of about 1000 cells per well. Using commercially available anti-isotypic reagents in an ELISA assay, the wells are screened for IgG2a production. Wells should be screened again by ELISA for IgG2a production and positive clones replated at 0.3 cells per well followed by another round of scr ning and replating at 0.3 cells per well. Approximately 1–5 out of $10^7$ cells which have switched isotype are optimally selected. Switches such as IgM to IgG can be done similarly using the appropriately coated antibody-coated beads. Accordingly, the isotype of TCSδ1 also referred to as δTCAR-3, described in U.S. Pat. application Ser. No. 115,256 filed Oct. 29, 1987, produced by hybridoma δTCR-3, deposited with the ATCC and assigned accession number HB9578, was switched from $IgG_1$ to $IgG_{2a}$ by the above method.

5.3. IMMUNOASSAYS

The antibodies of the invention, and fragments thereof containing the binding region (e.g., Fab, Fab', $F(ab')_2$), can be used in various immunoassays. Such immunoassays include but are not limited to competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, and immunoelectrophoresis assays, to name but a few.

5.4. DIAGNOSIS AND THERAPY

The antibodies of the invention, and fragments and derivatives thereof, can be valuable in the diagnosis and therapy of various conditions and disorders affecting the immune system.

5.4.1. DIAGNOSIS

In various embodiments, the antibodies, derivatives and fragments thereof of the invention can be used to detect, quantitate, and/or localize T lymphocytes which express TCRs that comprise and expose the defined region to which the antibody can bind. Both in vitro and in vivo assays can be used, including but not limited to the assays described in Section 5.3, supra. In addition, imaging techniques can be used, in which an antibody of the invention or derivative or fragment thereof is bound to a label. The labeled antibody can then be administered in vivo to determine the localization of its target antigen.

In particular embodiments of the invention, a lymphatic malignancy or immune disorder may be diagnosed by detecting the immunospecific binding of a monoclonal antibody, or derivative or fragment thereof, reactive with an epitope of a defined constant or variable region of a T cell antigen receptor in a patient sample. The patient sample may consist of any body fluid, including but not limited to peripheral blood, plasma, cerebrospinal fluid, lymphatic fluid, peritoneal fluid, or pleural fluid, to name but a few, or any body tissue. Binding may be accomplished and/or detected in vitro or in vivo. In vitro binding may be performed using histologic specimens or subfractions of tissue or fluid, i.e. substantially purified T cells. In vivo binding may be achieved by administering the antibody or fragment or derivative by any means known in the art (including but not limited to intravenous, intraperitoneal, intranasal, and intrasarterial, to name but a few) such that immunospecific binding may be detected; for example, by attaching a radioactive label to the diagnostic antibody, fragment, or derivative.

In specific embodiments of the invention, rheumatoid arthritis or Felty's syndrome may be diagnosed by detecting the binding of a monoclonal antibody, or fragment or derivative thereof, reactive with an epitope of Vδ1 (RA, FS), Vβ3, Vβ9, or Vβ10 (RA) variable region of a T cell antigen receptor in a patient sample, such that increased binding is detected in patients with rheumatoid arthritis or Felty's syndrome compared to normal controls.

In a preferred embodiment of the invention, rheumatoid arthritis or Felty's syndrome may be diagnosed by detecting binding of monoclonal antibody δTCS1, or fragments or derivatives thereof, in that the peripheral blood level of δTCS1 positive T cells in a patient with rheumatoid arthritis or Felty's syndrome is elevated compared to the level in the peripheral blood of a normal person. In a related embodiment, the percentage of total peripheral blood T cells which are δTCS1 positive is observed to be higher in a patient with rheumatoid arthritis or Felty's syndrome compared to the percentage of total peripheral blood T cells which are δTCS1 positive in a normal person. In additional embodiments of the invention, a diagnosis of rheumatoid arthritis or Felty's syndrome may be made based on detecting elevated numbers of δTCS1 positive T cells, relative to total T cells, in other body fluids and tissues including, but not limited to, synovial fluid and synovial membrane. In a further embodiment of the invention, rheumatoid arthritis or Felty's syndrome may be diagnosed in patients in which the peripheral blood ratio of δTCS1 positive to TCRδ1 positive T cells is elevated and, preferably, that it is greater than about 0.4. The binding of antibody or fragments or derivatives thereof may be detected in vitro or in vivo, as discussed supra. Intraarticar administration of a labelled antibody or derivative or fragments thereof may also potentially be utilized as a diagnostic procedure.

It should be understood that the diagnostic methods of the invention are best used in the context of other diagnostic parameters in order to obtain a comprehensive patient diagnosis. For example, a diagnosis of rheumatoid arthritis may be made based on the methods of the invention in the context of other clinical features of rheumatoid arthritis, such as typical joint involvement (chronic, symmetrical arthritis; early involvement most often in the hands); characteristic radiographic features; the presence of rheumatoid factor; the presence of rheumatoid nodules, etc. (Fishman et al., Medicine, Second Edition, J. B. Lippincott Company, Philadelphia, pp. 340–346). As with any diagnostic criteria, the parameters disclosed in the present invention may not be sole determinants, or pathognomonic, of a particular disorder.

Alternatively, according to the invention, a lymphatic malignancy or immune disorder may be diagnosed by detecting the presence of nucleic acid sequences homologous to a gene encoding a defined constant or variable region of a T cell antigen receptor in mRNA from a patient sample. Several procedures could be used to correlate TCR gene expression with disease. These involve 1) producing and analyzing cDNA libraries obtained from the disease related T cells to determine the presence of frequently used or

dominant TCR genes. 2) Analyzing disease samples by Southern blot to determine whether specific genetic polymorphisms (restriction fragment length polymorphisms, RFLPs) or oligoclonal TCR rearrangements exist. 3) Analyzing disease samples by the cDNA synthesis, polymerase chain reaction amplification, and slot blot hybridization procedure, see Section 11, infra. The third procedure represents a moreeficient procedure in the time required for analysis and in the number of patients that can be analyzed to detect a disease correlation. A fourth procedure using in situ hybridization of T cells without prior T cell culturing may also be extremely useful. Once the disease correlations of interest have been identified, then specific TCR based therapeutics, e.g. anti-TCR monoclonal antibodies, may be produced (see 11.3 infra).

In specific embodiments of the invention, rheumatoid arthritis may be diagnosed in a patient by detecting the presence of nucleic acid sequences homologous to a gene encoding Vδ1, Vβ3, Vβ9, or Vβ10 variable region of a T cell antigen receptor in mRNA from a patient sample, and finding that more frequently expressed Vβ genes include Vβ3, Vβ9, and VPβ10 and/or that a more frequently expressed Vδ gene is Vδ1.

5.4.2. THERAPY

In other embodiments of the invention, antibodies, derivatives, or fragments thereof directed against a defined region of the TCR can be therapeutically administered. For example, if the antibody is capable of inducing in vitro T cell proliferation, it may be administered therapeutically to stimulate specific cell-mediated immunity. In another particular embodiment, an antibody directed against a defined region of the TCR can be used to target a cytotoxic molecule to specific TCRs which are the causative agents of an autoiimmune disorder.

In another embodiment, an antibody may be administered therapeutically to block the interaction of effector T cells with their specific antigen and thus modulate a deleterious response. A further therapeutic embodiment is to administer an antibody therapeutically to bind to its target and mark those cells for elimination by the RES septem or by antibody dependent cell cytotoxicity (ADCC) reactions, the ablation of the target T cells resulting in a therapeutic effect. According to various embodiments of the invention, monoclonal antibodies directed toward defined regions of T cell antigen receptors may either be used therapeutically at low mitogenic concentrations to specifically activate the TCR bearing subset of interest, or alternatively could be used at much higher concentrations to bind to the receptors of interest, and thereby tag that T cell subset for elimination by the reticuloendotelial system. The important criteria for disease treatment is the ability to modulate specific disease related T cell subsets. The exact nature of this therapeutic modulation, whether to block or suppress a particular T cell subset, or alternatively, whether to stimulate and activate a particular subset, will depend upon the disease of interest and the specific T cell subset(s) involved.

According to various embodiments of the invention, the number of γδ+ T cells with cytotoxic activity may be increased by exposing T cells to an effective concentration of a monoclonal antibody, or derivative or fragment thereof, reactive with an epitope of the variable region of the delta chain of the T cell antigen receptor. Alternatively, higher concentrations of the same antibody may be effective in depleting γδ+ cells. The concentrations of antibody which induce proliferation, or which result in depletion, of γδ+ T cells may be accomplished by methods known in the art, as exemplified in Section 9 below. In specific embodiments of the invention, δTCS1 monoclonal antibody, or fragments or derivatives thereof, used at different concentrations, may result in proliferation, increased cytotoxic activity, or decreased cytotoxic activity, respectively, of T cell populations derived from patients with rheumatoid arthritis.

First generation treatments based on T cell receptor therapeutics may be developed based upon the correlation of specific TCR Variable region subfamilies with disease. These treatments offer an improvement over current procedures, such as anti-CD3 antibody treatment in patients undergoing renal transplant rejection, as the whole T cell population will not be modulated; only the particular T cell subset expressing the TCR V region subfamily of interest will be modulated. In addition, TCR variable region subfamily derived reagents are applicable to the treatment of groups of patients showing similar expression; whereas, a therapeutic combination based on a specific $V_\alpha$ (V-J-C) or a specific $V_\beta$ (V-D-J-C) may only be useful for the treatment of individual patients expressing that exact TCR combination.

Second generation TCR based therapeutics, may include further refinements of the association of particular variable (V), diversity (D) and joining (J) regions with specific disease states for α, β, γ and δ TCR genes. Patients may be further subdivided into groups for treatment based upon the TCR V, D, J and C regions involved; with the goal being to only modulate the actual disease related T cells and not to effect other T cell subsets.

In a particular embodiment of the invention, rheumatoid arthritis may be treated by administering therapeutically effective amounts of a monoclonal antibody, fragment or derivative thereof, which recognizes an epitope of the variable region of the beta chain of a T cell antigen receptor. In particular embodiments, the variable region comprises Vβ3, Vβ9, or Vβ10.

In additional embodiments of thenvention, a lymphatic malignancy or immune disorder may be treated by administering therapeutically effective amounts of a monoclonal antibody, or fragment or derivative thereof, which recognizes the constant region of the alpha chain or the variable region of the delta chain of a T cell antigen receptor. In one embodiment of the invention, rheumatoid arthritis or Felty's syndrome may be treated by administering therapeutically effective amounts of a monoclonal antibody, derivative, or fragment thereof, which recognizes an epitope of the Vδ1 variable region of the delta chain of a T cell antigen receptor. In a preferred embodiment, this monoclonal antibody has the binding characteristics of δTCS1. In a specific embodiment of the invention, a patient with rheumatoid arthritis may be treated by administering milligram quantities per day of δTCS1 antibody, or a fragment or derivative thereof, in a suitable pharmaceutical carrier into a patient in need of such treatment (See Section 10, infra).

5.4.3. THERAPEUTIC COMPOSITIONS; KITS

Various delivery systems are known and can be used for therapeutic delivery of the antibodies of the invention and fragments and derivatives thereof. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, and intranasal routes. In one embodiment, the antibody can be encapsulated in liposomes.

Various pharmacologic compositions may be utilized in order to deliver the antibodies, or fragments or derivatives thereof, according to the invention. Any suitable pharmaceutical agent with desirable solubility characteristics and chemical properties may be used, including but not limited to, where appropriate, saline or dextrose solutions.

Kits for practice of the instant invention are also provided. For example, such a diagnostic kit comprises in one or more containers a monoclonal antibody or derivative or fragment thereof reactive with an epitope of the constant region of the a TCR. In other embodiments, the antibody or derivative or fragment is reactive with Vβ5.3, or Vβ8.1 of the TCR. The antibody may be labeled. Alternatively, the kit can further comprise a labeled binding partner of the antibody, derivative or fragment. Therapeutic kits can comprise the therapeutic compositions of the invention in one or more containers.

5.5. MONOCLONAL ANTIBODIES REACTIVE WITH THE CONSTANT REGIONS OF THE T CELL ANTIGEN RECEPTOR ALPHA POLYPEPTIDE

In a specific embodiment, the present invention relates to monoclonal antibodies reactive with the constant region of the alpha chain of the TCR. In a particular example, the invention relates to mAb αF1. In another particular embodiment, the invention relates to mAb αF2.

αF1 and αF2 recognize two different epitopes of the framework region of the constant region of the TCRα monomer. In various embodiments of the invention, αF1 or αF2, or both, or fragments or derivatives of either, or both, can be used to bind to TCRα peptide amino acid sequences, either as part of an intact TCR complex or α peptide, or a fragment thereof.

Monoclonal antibodies αF1 and αF2 are described more fully in the subsections below and in Section 6, infra.

5.5.1. BINDING SPECIFICITIES OF αF1 AND αF2

Monoclonal antibodies αF1 and αF2 were found to react with αβ TCR T cell lines, but did not-react with αβ $TCR^-$T cell lines (including γδ$TCR^+$ cell lines) or B cell lines. Since αF1 and αF2 react with TCR on many T cell lines and a large percentage of PBLs, they are reactive with major framework regions of the TCR molecule.

Both mAb αF1 and αF2 immunoprecipitate the 90 kD αβ TCR heterodimer. mAb αF1 reacts with the oligopeptide representing amino acid residues 141–159 of the a peptide, whereas αF2 reacts with the oligopeptide representing amino acid residues 212–231. Both oligopeptides reside within the constant region of the a chain sequence. Both αF1 and αF2 selectively immunoprecipitated in vitro synthesized peptide.

5.5.2. IMMUNOHISTOLOGICAL ANALYSIS USING αF1 AND αF2

Although both αF1 and αF2 immunoprecipitate TCR of cell lysates of diverse T cell lines, neither bind to viable T cells. Presumably this is because the epitopes recognized by αF1 and αF2, on the constant region of α chain TCR, are hidden on the cell surface.

When the integrity of the cell membrane is disrupted, for example, by histologic fixative, αF1 and αF2 can be used to bind to TCR α peptide. By immunohistological staining, both αF1 and αF2 recognize greater than 90% of both mature peripheral resting and activated T cells, corresponding to the distribution of αβ TCR in the adult T cell population.

5.5.3. USES OF αF1 AND αF2

Because αF1 and αF2 bind to epitopes within the constant region of TCR α peptide, both mAb can be used to define populations of TCR $\alpha^+$ cells.

For example, in one embodiment of the invention αF1 and αF2 can be used to study TCRα expression during thymocyte differentiation. It is thought that thymocytes differentiate and mature gradually as they pass from thymic cortex to medulla and finally to peripheral blood (Benjamini and Leskowitz, 1988, *Immunology A Short Course*, Alan R. Liss, Inc., New York p. 141). Numerous studies have suggested that in thymus, during T cell differentiation, δ, α, and γ chain genes are rearranged and transcribed earlier than α chain genes (Chien et al., 1984, Nature 312:31; Raulet et al., 1985, Nature 314:103; Snodgrass et al., 1985, Nature 315:332). αF1 and αF2 can be used to correlate the expression of TCR α protein with thymocyte development.

Furthermore, αF1 and αF2 can be used to determine the state of αTCR protein expression in a cell. For example, unstimulated CEM cells express neither TCR nor CD3 on their surface; however, stimulation with PMA induces CD3 expression (Schackelford et al., 1987, J. Immunol, 138:613). Using αF1, the unstimulated CEM line was found to be negative for the expression of α chain TCR protein in the cytoplasm; however, stimulation with PMA resulted in an increase in intracellular TCRα and surface expression.

αF1 and/or αF2 can also be used to generate or select monoclonal antibodies directed at unique epitopes of the TCRα constant region, i.e. by competitive binding studies. Novel mAb reacting with different epitopes of the TCR α constant region would not compete with αF1 or αF2 for TCR α binding. αF1 and αF2 can also be used to generate antibodies to the αTCR constant region that are able to react with viable cells.

αF1 and αF2 can also be valuable in diagnosis and therapy, as described infra.

In one embodiment of the present invention, αF1 and/or αF2 can be used to detect TCR α protein expression in specimens in vitro, e.g., in cell lysates or in histologically prepared specimens. In one particular embodiment, classification of lymphatic malignancies into B cell, T cell, or non-B non-T cell groups, can be greatly facilitated by demonstrating the presence or absence of TCR α protein using αF1 and/or αF2 according to the present invention.

αF1 and αF2, in various embodiments of the present invention, can be used to establish the cellular derivation of lymphatic malignancies, such as leukemia, lymphoma, and myeloproliferative diseases, and may prove useful in the diagnosis of other malignancies or non-malignant disorders of the immune system.

In another embodiment of the present invention, αF1 and αF2 can be used to monitor therapies involving the proliferation or ablation of $\alpha^+$T cells.

In a further embodiment of the present invention, αF1 and αF2 could be used to detect TCR-a-producing tissues in vivo. Because αF1 and αF2 do not bind to viable cells, binding would only occur to cells which exhibit enhanced permeability, such as moribund or necrotic cells. These cells would, most likely, be localized to malignant tumors or similar neoplasms associated with lymphoid cell death. In an embodiment of the invention, αF1 and αF2 could be conjugated to a label comprising, for example, a radioisotope. Labeled αF1 and αF2 could then be injected into a patient; localization of radiolabeled antibody can reveal tumor sites.

5.6. MONOCLONAL ANTIBODIES REACTIVE WITH THE VARIABLE REGION OF THE T CELL ANTIGEN BETA POLYPEPTIDE

In another specific embodiment, the present invention relates to monoclonal antibodies reactive with specific variable regions of the beta chain of the TCR. In one particular embodiment of this aspect of the invention, the invention relates to a monoclonal antibody reactive with Vβ5.3. An example of such a Vβ5.3-specific antibody is mAb W112. In another particular embodiment, the invention relates to a monoclonal antibody reactive with Vβ8.1. An example of such a mAb, reactive with human Vβ8.1, is mAb 2D1.

In various embodiments of the invention, W112 or 2D1, or fragments or derivatives of either, can be used to bind

with TCRβ peptide amino acid sequences, either as part of an intact TCR complex or β peptide, or T cell surface protein, or a fragment thereof. Monoclonal antibodies W112 and 2D1 are described more fully in the subsections below and in Section 7, infra.

5.6.1. BINDING SPECIFICITIES OF W112 AND 2D1

W112 immunoprecipitates a heterodimer of 48 kd and 40 kd from HPB, and 2D1 immunoprecipitates the same size dimer from Jurkat cell lysates. Both W112 and 2D1 are anti-minor framework antibodies as they react with a minority of peripheral blood T cells, indicating specificity directed toward the variable portion of TCRβ. Western blot analysis showed that both W112 and 2D1 reacted with the TCRβ protein of their respective cell lines. W112 is a TCR Vβ5.3 specific reagent and 2D1 is a TCR Vβ8.1 specific reagent.

5.6.2. USES OF W112 AND 2D1

Because both W112 and 2D1 are anti-minor framework TCRβ specific reagents , these mAb can be used to study, define, quantitate, and localize a subset of T cells.

In one embodiment of the present invention, W112 and 2D1 can be used to study Vβ5.3 and Vβ8.1 expression during thymocyte differentiation. In another embodiment, W112 and 2D1 can be used to generate or select additional anti-minor framework antibodies, i.e., by competitive binding studies.

W112 and 2D1 can also be valuable in diagnosis and therapy, as described infra.

In one embodiment, W112 and/or 2D1 can be used to detect Vβ5.3 and Vβ8.1 subsets of T cells in specimens in vitro, e.g., in cell lysates or in histologically prepared specimens. Such use is of value in the diagnosis of patients with immune system disorders affecting such subsets of their T lymphocytes. In an embodiment of the present invention, W112 and 2D1 can be used to monitor therapeutic procedures that effect these subsets of T cells.

Since both W112 and 2D1 react with viable cells, they can be used therapeutically to modulate Vβ5.3 and Vβ8.1 expressing T cells involved in disease. In one embodiment, W112 or 2D1 could be administered therapeutically to ablate the Vβ5.3 or Vβ8.1 T cell subsets. In another embodiment, W112 or 2D1 could be administered therapeutically to induce the proliferation of the Vβ5.3 or Vβ8.1 T cell subsets. In these embodiments the selective ablation or proliferation of a specific T cell subset by anti-minor framework antibodies will be preferred to modulating the whole T cell population with anti-major framework or pan-T reagents, as only the disease specific T cell subsets will be effected.

In another embodiment of the present invention, W112 or 2D1 could be conjugated to a label comprising, for example, a radioisotope. Labeled W112 or 2D1 could then be injected into a patient; localization of radiolabeled antibody can reveal Vβ5.3 or Vβ8.1 specific disease sites.

## 6. EXAMPLE: THE GENERATION OF TWO MONOCLONAL ANTIBODIES αF1 AND αF2, DIRECTED AGAINST TWO DIFFERENT EPITOPES OF THE HUMAN αTCR CONSTANT REGION

We describe herein two mAb, termed αF1 and αF2, generated against purified αβ heterodimer TCR. Using synthetic oligopeptides, we demonstrated that αF1 recognized amino acid residues 141–159, and αF2 recognized amino acids 212–231, of the constant region of the α chain TCR. Although neither mAb reacted with viable T cells, both antibodies immunoprecipitated TCR αβ heterodimer from HPB, Jurkat, and PBL cells, α chain protein of PMA-stimulated CEM cell line, and a 32 kD in vitro translation product of a chain cDNA. These antibodies have proved to be useful in the immunohistochemical staining of human tissues. These two mAb are valuable tools in the study of TCR and in the clinical classification of T cell lineage neoplasms.

6.1. MATERIALS AND METHODS

6.1.1. CELL LINES

The human cell lines HPB-ALL, Jurkat, Daudi, U923, CEM and Molt-4 were obtained from the American Type Culture Collection (Rockville, Md.), and were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum. Sequence analyses of the HPB-ALL and HPB-MLT has indicated that both cell lines have the same β and α TCR genes and may have been confused during in vitro culturing (Berkhout et al., 1988, Nucleic Acids Research 16:5209). These cells are referred to as HPB cells to indicate that they are the same line. The cell line Molt-13 was supplied by Dr. Michael Brenner, Harvard Medical School, Boston, Mass. TIL21 and TIL21pBT are human T cell lines isolated from infiltrated lymphocytes of lung tumor (Flatow et al., 1988, FASEB J. 2:3505). Human peripheral blood lymphocytes (PBL) were isolated from normal donors using Ficoll-Hypaque gradient separation (Pharmacia, Piscataway, N.J.).

6.1.2. MONOCLONAL ANTIBODIES

The murine hybridomas 3A8 and 3D6 were generated by immunizing eight week old female Balb/c mice (Jackson Laboratories, Bar Harbor, Me.) with 1.0 μg of purified αβ TCR protein in complete Freund's adjuvant intraperitoneally. At two-week intervals the mice were given 1.0 μg purified αβ TCR in incomplete Freund's adjuvant intraperitoneally. After three months, sera were tested to be positive as determined by immunoprecipitation of TCR heterodimer using $^{125}$I-labelled HPB and Jurkat cell lysates. Four days before fusion, the mice received the final intravenous injection of 1 lg of purified TCR in PBS. Hybridoma cells were generated using an established protocol (Wu et al., supra). The mAb produced by clones 3A8 and 3D6 were designated as αF1 and αF2, respectively. The isotypes of mAb αF1 and αF2 were determined to be IgG2a and IgG2b, respectively, using a commercial typing kit (Zymed, San Francisco, Calif.). βF1 (Brenner et al., 1987, J. Immunol. 138:1502), and δTCS-1 (Wu et al., supra.) (T Cell Sciences, Cambridge, Mass.) are mAb that react with the β and δ chains of TCR, respectively. OKT3 was purchased from Ortho Diagnostic System (Raritan, N.J.)

6.1.3. T CELL LYSATES AND αβ TCR PROTEIN

Human T cell lines bearing the appropriate TCR were solubilized at $5\times10^7$ cells/ml in lysis buffer containing 10 mM Tris PH 8.0, 1% Nonidet -P 40 (NP-40), 10 mM iodoacetamide (IAA), 1 mM phenylmethyl sulfonyl fluoride (PMSF), 0.04% aprotinin and 0.3 mM N-tosyl-L-phenylalanine chloromethyl ketone (TPCK). HPB cell lysates were applied to a mixed lectin column of lentil- and ricin-agarose column (Sigma, St. Louis, Mo.). The effluent from the lectin column was further purified through a column of mAb βF1 immobilized Affi-gel 10 (Biorad, Rockville Centre, N.Y.). The bound αβ TCR protein was then eluted with solution containing 25 mM of diethylamine, pH 11.5 and 0.2% NP-40. In general, approximately 80 μg of a TCR protein were purified from $5\times10^{10}$ HPB-ALL cells.

6.1.4. ELISA METHOD FOR SCREENING mAb (FIG. 6A)

96-well microtiter plates (Dynatech, Alexandria, Va.) were coated overnight at 4° C. with 100 μl per well of goat anti-mouse IgG Fc-specific antibodies (Cappel, West Chester, Pa.) at 1 μg/ml in PBS. This was designed to preferentially select for monoclonal antibodies having an

IgG isotype. Plates were then blocked with 100 μl per well of 1% BSA in PBS containing 0.05% Tween-20 for 30 minutes. 100 μl of hybridoma supernatants were added to each well and incubated for 1 hour. 100 μl of cell lysate containing 25% FCS and 0.1 μg/ml of biotin-labelled βF1 $F(ab)_2$ fragment were then added to each well and incubated for an additional 2 hours. After washes, 100 μl horseradish peroxidase (HRP) conjugated streptavidin (Zymed, San Francisco, Calif.) was added at 1:2500 dilution to each well. After 30 minutes of incubation, 50 μg of the 0.1% O-phenylenediamine dihydrochloride (OPD) (Sigma, St. Louis, Mo.) substrate solution was added to each well.

The reactions were stopped with 100 μl of 2N $H_2SO_4$. The absorbance of each well was then read on a micotiter plate reader (Dynatech, Cambridge, Mass.) at 490 nm. Between each step, ELISA plates were washed four times with 250 μl per well of PBS containing 0.5% Tween-20 and the incubations were carried out at room temperature.

6.1.5. ASSAY FOR TCR PEPTIDE BINDING

TCR oligopeptides were custom synthesized by Pennisular Laboratory (Belmont, Calif.) according to the deduced sequences from the α-and β-chain TCR genes (Yanagi et al., 1985, Proc. Nat;. Acad. Sci. U.S.A. 82:3430; Yanagi et al., 1984 Nature 308:145–149). TCR oligopeptides were coupled to protein carrier, BSA (Sigma, St. Louis, Mo.) with succinimidyl-4-(p-maleimidophenyl) butyrate (Pierce, Rockford, Ill.) according to the published procedure (Gitman et al., 1985, Proc. Natl. Acad. Sci. U.S.A. 82:7309). 100 μl of 4.0 μg/ml the peptide conjugates were added per well to microtitre plates and incubated overnight at 4° C. Plates were blocked with PBS containing 1% BSA and 0.05% Tween-20 for 30 minutes. 100 μl of each of the test mAb at about 10 μg/ml were added to each well and incubated for 2 hours. After washing, 100 μl per well of 1:1000 dilution of HRP-conjugated goat anti-mouse IgG antibodies (Zymed, San Francisco, Calif.) were added to each well of the plate and incubated for an additional two hours, followed by the addition of 0.1% of OPD substrate solution. The reactions were stopped with 100 μl of 2N $H_2SO_4$. The absorbance of each well was then read on a microtiter plate reader (Dynatech, Cambridge, Mass.) at 490 nm. Between each step, ELISA plates were washed four times with 250 μl per well of PBS containing 0.5% Tween-20 and the incubations were carried out at room temperature.

6.1.6. IMMUNOPRECIPITATION AND SDS-PAGE

Cells were harvested at log growth phase and their surface proteins were labelled with $^{125}I$ by the lactoperoxidase method (Brenner et al., 1984, J. Exp. Med. 160:541). Lysates of the iodinated T cells were prepared in a manner similar to that described above. Immunoprecipitation and one dimensional SDS-PAGE using 10% gels under either non-reducing or reducing conditions were performed as previously described (Wu et al., supra).

6.1.7. IMMUNOHISTOLOGICAL TISSUE SECTION ANALYSIS

Culture cells were cytocentrifuged on glass slides (Cytospin, Shandon, Pittsburgh, Pa.) and fixed with 100% methanol. The immunoperoxidase staining of cytospun cell smears was carried out using commercial staining kits (CRL, Cambridge, Mass.). Immunoperoxidase staining was also carried out on frozen cryostat sections of thymus and tonsil.

6.1.8. IN VITRO TRANSLATION

Phage Sp6 RNA polymerase and rabbit reticulocyte in vitro translation system were purchased from New England Biolabs (Beverly, Mass.) and Bethesda Research Laboratories (Gaithersburg, Md.), respectively. pGA5-pSP73 plasmid construct was a gift of Dr. Michael Brenner, Harvard Medical School, Boston, USA. pGA5-pSP73 is a plasmid construct composed of a pSP73 plasmid backbone (Promega, Wis., USA) and a coding region for the α chain TCR gene derived from the pGA5 cDNA clone (Sim et al., 1986, Nature 312:771). pGA5-pSP73 plasmid construct was linearized with restriction enzyme XhoI, and was subsequently used as the DNA template for the transcription of the pGA5 α chain gene using the phage Sp6 promoter (Krieg et al., 1984, Nucl. Acids Res. 12:7057; Hope and Struhl, 1985, Cell 43:177). The resulting capped pGA5 RNA transcript was then translated into $^{35}S$-labeled α chain protein in the rabbit reticulocyte in vitro translation system according to the manufacturer's protocol. Rabbit globin mRNA was also included in the in vitro translation system as an added irrelevant protein for the immunoprecipitation assays.

6.2. RESULTS

6.2.1. ISOLATION OF MAJOR FRAMEWORK mAb.

Both mAb αF1 and αF2 tested positive in the ELISA using cell lysates of $\alpha\beta TCR^+$ HPB and Jurkat cell lines, but tested negative with cell lysates of MOLT-4 and Daudi. MOLT-4 is a T cell line that lacks α chain TCR and Daudi is a B cell line that is αβ TCR negative. The Nabs were further analyzed by immunoprecipitation using $^{125}I$-labeled HPB and MOLT 13 cell lysates. Both mAb immunoprecipitated a 90 kD protein of TCR α and β heterodimer from HPB cells under non-reducing conditions (FIG. **1***a*, lanes 1 and 2). The same αβ TCR protein is immunoprecipitated by βF1, a mAb directed against β framework of TCR (FIG. **1A**, lane 3) (Brenner et al., 1987, J. Immunol. 138:1502). Under reducing conditions, both αF1 and αF2 mAb immunoprecipitated two proteins of 40 and 49 kD from HPB cells, as does βF1 (FIG. 1B, lanes 1, 2 and 3). In contrast, no proteins were immunoprecipitated by either αF1, αF2, or βF1 when membrane lysates from Molt-13, a γδ T Cell line, was used (FIG. 1A and **1B**, lanes 5, 6, 7).

As a control, the Vδ specific antibody, δTCR-1, immunoprecipitated the γδTCR from the $\gamma\delta^+$ cell line MOLT-13, but not from the αβ cell line HPB (FIG. 1A and **1B**, lanes 4 and 8).

Similar results were also obtained with cell lysates isolated from $^{125}I$-labeled $\alpha\beta^+$ Jurkat cells and PBLs (FIG. **2**).

6.2.2. REACTIVITY OF mAb αF1 AND αF2

Immunocytochemical staining using αF1 and αF2 mAb was performed on a variety of cell lines. As shown in Table 1, both αF1 and αF2 mAb reacted positively with αβ T cell lines HPB, Jurkat, TIL21 and TIL21PBT, but negatively with γδ T Cell line Molt-13 and non-T cell lines Daudi and U923. Also αF1 and αF2 detected the presence of α chain TCR protein in the PMA stimulated CEM line, but not in the unstimulated cells.

TABLE 1

CROSS REACTIVITY OF mAb WITH DIFFERENT HUMAN CELL LINES

| | | Reactivity with mAb | | | | |
|---|---|---|---|---|---|---|
| Cell Name | Cell Type | OKT3 | BF1 | αF1 | αF2 | δTCS1 |
| HPB | T Cell | ++ | +++ | +++ | +++ | – |
| Jurkat | T Cell | ++ | +++ | +++ | +++ | – |
| TIL21 | T Cell | ++ | ++ | ++ | ++ | – |
| TIL21pBT | T Cell | ++ | ++ | ++ | ++ | – |
| CEM | T Cell | + | ++ | – | – | – |
| CEM + PMA** | | ++ | ++ | ++ | + | – |

TABLE 1-continued

CROSS REACTIVITY OF mAb WITH DIFFERENT HUMAN CELL LINES

| | | Reactivity with mAb | | | | |
|---|---|---|---|---|---|---|
| Cell Name | Cell Type | OKT3 | BF1 | αF1 | αF2 | δTCS1 |
| Molt-13 | T Cell | ++ | – | – | – | + |
| Daudi | B Cell | — | — | — | — | — |
| U923 | Monocyte | — | — | — | — | — |

*Cell growing at log phase were washed and cytospinned onto glass slides. Immunohistological staining with different mAb was performed as described. Reactivity was determined arbitrarily on a scale of – to +++. **CEM cells were incubated with PMA at 1 ng/ml for three days before being harvested and stained with mAb.

Both αF1 and F1 were used to stain cryostat sections of human thymus and tonsil. As shown in FIG. **3**, αF1 stained about 70% of T cells in the cortical zone (3A) and over 90% in the medullary zone (3C) of the thymus. αF1, however, stained about 30% T cells in cortex (3B) and 90% in medulla (3D). When tested on tonsil tissue section, both mAb reacted with T cells in the interfollicular areas (E and F). αF2 staining was similar but weaker than that of αF1. Neither mAb stained viable T cells at a detectable level.

6.2.3. SPECIFICITY OF αF1 AND αF2 mAb

To further define the specificity of αF1 and αF2, both mAb were tested for their ability to recognize synthetic oligopeptides derived from α-chain TCR sequence in an ELISA. The reactivities of αF1 and αF2 against α and β chain derived synthetic oligopeptides are shown in Table 2.

TABLE 2

REACTIVITY OF αF1 and αF2 WITH SYNTHESIZED α AND β PEPTIDES

| | BOUND ($OD_{490}$) MONOCLONAL ANTIBODY | | | |
|---|---|---|---|---|
| Peptide* | αF1 | αF2 | βF1 | δTCS1 |
| α21–42 | 0.04 | 0.08 | 0.04 | 0.04 |
| α141–159 | 0.26 | 0.05 | 0.08 | 0.04 |
| α163–182 | 0.04 | 0.05 | 0.05 | 0.05 |
| α212–231 | 0.03 | 0.86 | 0.06 | 0.05 |
| β42–56 | 0.04 | 0.07 | 0.04 | 0.04 |
| β144–161 | 0.05 | 0.05 | 0.04 | 0.06 |
| β192–210 | 0.08 | 0.04 | 0.07 | 0.03 |
| β231–244 | 0.06 | 0.04 | 0.06 | 0.04 |

*α and β peptides from both the variable and constant region were coupled to protein carrier and coated on the microtitre plates. Reactivity with mAb was observed by developing a signal with a second goat anti-mouse-HRP conjugate. The number after α and β indicate their spanning location residue within their corresponding α and β TCR chains.

mAb αF1 reacted with the oligopeptide derived from TCR α chain spanning from amino acid 141 to 159 (α141–159) whereas mAb αF2 reacted with a different oligopeptide derived from TCRα amino acid 212 to 231 ($\alpha^{212-231}$). Both oligopeptides reside within the constant region of the α chain sequence. Six other oligopeptides derived from other regions of α and β chains of human TCR did not react with either αF1 or αF2. Neither of the control antibodies, βF1 and δTCS-1, reacted with the synthetic α or β chain oligopeptides.

In addition, in a competitive TCR-ELISA immunoassay, oligopeptide α141–159 blocked the binding of mAb αF1 to TCR isolated from HPB, with a $K_d$ of $2\times10^{-9}$ M (FIG. **4***a*). Similarly, synthetic oligopeptide α212–231 was able to compete with the TCR of HPB for the binding to αF2 ($K_d 5\times10^{-9}$M) (FIG. **4***b*). None of the other oligopeptides tested was able to compete with HPB TCR in the TCR-ELISA assay.

6.2.4. IMMUNOPRECIPITATION OF IN VITRO SYNTHESIZED α CHAIN TCR BY αF1 AND αF2

To further confirm the specific reaction of αF1 and αF2 with α chain TCR protein, both mAb were used in an immunoprecipitation assay using an in vitro translated protein mixture containing a known α chain TCR protein and an irrelevant protein, rabbit globin (FIG. **5**, Lane 6). In this assay, αF1 and αF2 specifically immunoprecipitated an chain TCR protein of 32 kD but not the irrelevant rabbit globin (12 kD)(FIG. **5**, Lane 1 and 2). In contrast, there were no proteins immunoprecipitated when two isotype matched, irrelevant mAb were used (FIG. **5**, Lane 3 and 4).

6.3. DISCUSSION

We have generated two mAb, αF1 and αF2, directed against two distinct epitopes on the constant region of the α chain of TCR protein. Both mAb were shown to immunoprecipitate an αβ heterodimer of human TCR from αβ T cell lines, such as HPB and Jurkat, as well as peripheral blood lymphocytes. By immunohistological staining, both antibodies recognized greater than 90% of both the mature peripheral resting and activated T cells and lymphocytes in thymus and tonsil. Both mAb also immunoprecipitated the in vitro translated product of an a cDNA clone. Using synthetic oligopeptides prepared according to the published sequences of α chain constant region, we have identified the specific epitopes recognized by both mAb αF1 and αF2.

Both αF1 and αF2 do not stain viable T cells. It is surprising that even αF1, which recognized an epitope in the constant region that was very close to the variable region is not able to recognize the surface TCR proteins on viable T cells. Thus, it appears that the epitopes recognized by αF1 and αF2 on the constant region of α chain TCR are hidden on the cell surface. It should also be noted that αF1 stains all the available T cells expressing the α chain TCR protein. This observation indicates that the epitope recognized by αF1 is conserved among all the αβ T cells.

TCR is present on the cell surface as a complex associated with at least three other proteins, the CD3 complex (Davis and Bjorkman, 1988, supra). Both the constant region of the αβ TCR and the CD3 complex are highly non-polymorphic. This conservation of sequence suggests that the constant region of TCR may serve as an important functional domain to interact with the CD3 complex for the transduction of signals during T cell activation. Upon binding of antigen, the variable region of TCR may undergo conformational changes and subsequently alter the interaction between the constant region of TCR and the CD3 complex. The latter alteration may thus initiate the functional role of the CD3 complex in snal transduction during T cell activation. αF1 and αF2 will be useful in further analyzing this process.

Both αF1 and αF2 are useful for studying TCR biosynthesis during T cell differentiation. αF1 identifies T cells in tissue section, as does βF1. Numerous studies have suggested that in the thymus, during T cell differentiation, TCR δ, γ and β chain genes are rearranged and transcribed earlier than α chain genes (Chien et al., 1984, Nature 312:31; Raulet et al., Nature 314:103; Snodgrass et al., 1985, Nature 315:232). It is also suggested that in thymus T cells differentiate and mature gradually along their path from cortex to medulla and finally to peripheral blood. The differential staining patterns of αF1 versus βF1 in the cortical area of the thymus may indicate the presence of such a process of T cell maturation.

αF1 and αF2 can also be used to study TCRα protein expression. It has been reported that in the absence of α chain TCR, the CEM cell line does not express TCR and CD3 complexes on its cell surface (Uppenkamp et al., 1988,

J. Immunol. 140:2801). However, upon stimulation with PMA, the CEM line does express surface CD3 complexes (Shackelford et al., 1987, J. Immunol. 138:613). Using αF1, we found that the unstimulated CEM line is negative for the expression of α chain TCR protein. However, in the presence of PMA, the intracellular level of α chain TCR protein increases and the TCR:CD3 complex appears on the cell surface. This observation demonstrates that PMA induces the expression of α chain protein, resulting in surface CD3 expression.

αF1 and αF2 can also be useful in the clinical classification or diagnosis of lymphoid malignant or non-malignant disease. In mouse, it has been demonstrated that the expression of β protein precedes that of a protein. It is, therefore, possible to identify undifferentiated leukemic cells where the various stages of differentation can be subclassified. Thus, αF1 and αF2 are useful reagents in analyzing the pathway of TCR expression in man and may also be valuable reagents for the clinical classification of T lymphocytes in disease states.

Similarly, abnormal expression of TCRα in malignant cells can be detected by αF1 and αF2. The T-cell lymphoma cell line SUP-T1 (Denny et al., 1986, Nature 320:549) expresses a hybrid mRNA transcript comprised of an immunoglobulin heavy-chain variable region and a portion of TCRα, including J segment sequences. αF1 and αF2 were used to show that this hybrid mRNA is, in fact, translated into a protein which includes a portion of the TCRα constant region.

## 7. EXAMPLE: MONOCLONAL ANTIBODIES TO THE VARIABLE REGIONS OF HUMAN T CELL ANTIGEN RECEPTOR

### 7.1. RESULTS

#### 7.1.1. GENERATION OF W112 AND 2D1 MONOCLONAL ANTIBODIES

To make mAb specific to VPβ5.3 and Vβ8.1, two human T cell leukemic lines, HPB and Jurkat, were used as immunogens. HPB expresses Vβ5.3 on its cell surface (Leiden and Strominger, 1986, Proc. Natl. Acad. Sci. USA 83:4456–4460; Leiden et al., 1986, Mol. Cell Biol. 6:3207–3214; Toyonaga and Mak, 1987, Ann. Rev. Immunol. 5:585–620), and Jurkat cells express Vβ8.1 (Yanagi et al., 1984, Nature 308:145–149; Leiden and Strominger, 1986, supra; Toyonaga and Mak, 1987, supra). BALB/c mice were immunized with $1\times10^7$ HPB-MLT or Jurkat cells. Four weeks later, the same amount of cells were injected intravenously into the primed mice and the splenocytes were fused after four days with mouse myeloma P3X63Ag8.653 cells. Ten days later, hybridomas were screened for anti-TCR activity with an ELISA protocol as illustrated in FIG. **6**(*a*), using HPB and Jurkat cell lysates. Positive wells were cloned by limiting dilution and clones rescreened. Data from a sample ELISA rescreening is shown in Table 3. Out of 500 hybridoma supernatants screened, only 2D1 was positive in the ELISA assay. Anti-TCR major-framework mAb, W76, was used as a positive control as it binds to the constant region of every β chain. Anti-CD3 mAb, OKT3, was used as the negative control.

TABLE 3

| Screening: | 2D1 | W76 | OKT3 |
|---|---|---|---|
| 1 | 0.132 | 0.165 | 0.055 |
| 2 | 0.278 | 0.315 | 0.115 |

W76 is an anti-TCR major framework reagent used as a positive control. OKT3 is an anti-CD3 reagent used as a negative control.

To further confirm the TCR-ELISA screening results, CD3-comodulation assays were run as shown in FIG. **6**(*b*). For the comodulation assays, Jurkat or HPB cells were incubated overnight with hybridoma supernatants. Cells were then washed and reincubated with FITC-conjugated OKT3, and analyzed by flow cytometry. Positive hybridoma supernatants such as 2D1 that were able to modulate the TCR-CD3 complex off of the cell surface, stained negatively with OKT3 the following day. Negative hybridoma supernatants stained positively with OKT3 the following day. From the screening, mAb W112 reactive with HPB and 2D1 reactive with Jurkat were identified.

#### 7.1.2. SPECIFICITY OF W112 AND 2D1

The reactivity of W112 and 2D1 with TCR on HPB and Jurkat cells was tested using immunoprecipitation. $^{125}$I surface labelled HPB and Jurkat cell lysates were incubated with mAb and the precipitated immune complexes were then run on 10% SDS-PAGE (FIG. **7**). W112 specifically immunoprecipitated a heterodimer of 48 kD and 40 kD from HPB lysates (FIG. **7**A). 2D1 immunoprecipitated the same sized heterodimer from Jurkat cell lysates (FIG. **7**B). The molecular weights of the immunoprecipitated proteins are similar to those precipitated by βF1, a mAb reactive with a framework epitope of the β chain constant region, or by αF1, the anti-major framework anti-TCRα antibody (Section 6, supra).

The reactivity of W112 was also tested by Western blot analysis (FIG. **8**). HPB cell lysates were fractionated by 10% SDS-PAGE and then transferred to nitrocellulose sheets. The sheets were first incubated with W112, washed and then incubated with horse radish peroxidase (HRP) conjugated goat anti-mouse antibody. After washing, color was developed with an HRP substrate. W112 specifically reacted with a 40 kD protein. This 40 kD protein also reacted with the β constant region reagent βF1 (FIG. **8**) indicating W112 was TCRβ chain specific.

To determine the chain specificity of 2D1, Jurkat cells were metabolically labelled with $^{35}$S-methionine and the cell lysates were incubated with 2D1. Immunoprecipitated complexes were then fractionated by 10% SDS-PAGE and the dried gel autoradiographed. Antibodies 2D1 and βF1 reacted with the 40 kD β chain of Jurkat cells (FIG. **9**).

#### 7.1.3. FLUORESCENT ACTIVATED CELL SORTING USING W112 AND 2D1

Reactivity of W112 and 2D1 was determined by FACS analysis. PBLs from normal donors were reacted with W112, 2D1, OKT3 or normal mouse serum, washed, incubated with FITC-labelled goat anti-mouse antibody and analyzed on an Ortho® flow cytometer (FIG. **10**). When tested against normal PBLs, W112 and 2D1 antibodies detected only minor populations of cells. Among 10 samples tested, W112 reacted with from 0.3% to 5% peripheral blood cells, and 2D1 reacted with 0.5% to 13%. The positive control antibody, OKT3, represented a pan-T cell reagent, and stained 70% of the peripheral blood lymphocytes. The negative control reagent, normal mouse serum, was non-reactive. Since W112 and 2D1 react with subsets of T cells, they are anti-minor framework antibodies.

7.2. DISCUSSION

Together these results indicate that both W112 and 2D1 antibodies detect a small subset of T cells in the large population of cells present in peripheral blood. W112 and 2D1 are TCR anti-minor framework specific reagents that react with only that subpopulation of calls in peripheral blood that expresses that particular minor framework determinant protein. Both antibodies are specific to the β chain of the TCR heterodimer and furthermore are specific to that variable region present on the cells used as their immunogen.

2D1 is a TCR Vβ8.1 specific reagent. W112 was determined to be a TCR Vβ5.3 specific reagent by the following criteria. It has been reported that the T cell leukemia line HPB-MLT rearranges two TCR β genes which are expressed at the mRNA level (Leiden, et al., 1986, Molec. Cell. Biol. 6:3207). Cloning and sequencing of cDNA clones corresponding to the two rearranged genes revealed that one of the rearrangements is defective and is characterized by a out-of-frame V-D-J joining event which causes several stop codons in downstream C-region sequences. A second cDNA represents the functional β gene from HPB since it is composed of a full-length open reading frame and its deduced amino acid sequence corresponds to the amino-terminal protein sequence of the β chain isolated from HPB-ALL cells (Jones, et al., 1985, Science 227:311. The deduced amino acid sequence for the defective gene is identical to the V region sequence which identifies the Vβ6.1 family (Concannon, et al., 1986, Proc. Natl. Acad. Sci. 83:6598; Kimura et al., 1986, J. Exp. Med. 164:739). The amino acid sequence for the functional gene is identical to the V region sequence which identifies the Vβ5.3 family. Thus, TCR Vβ-specific antibodies which bind to HPB cells must recognize the Vβ5.3 family. Thus, W112 is a TCR Vβ5.3 specific reagent.

W112 and 2D1 were generated against specific T cell clones having defined TCR proteins and selected for the preferred IgG isotype using the TCR ELISA. Different IgG subtypes for these antibodies could be selected by the isotype switching protocol described in Section 5.2, supra. These reagents have clinically relevant uses in disease diagnosis and therapy. Since these antibodies react with anti-minor framework determinants present on specific T cell subsets, they would be preferred clinical reagents for detecting or modulating Vβ5.3 and Vβ8.1disease specific T cells as compared to anti-major framework reagents or pan-T reagents that by their nature must affect large populations of T cells.

The data presented supra for generating TCR antibodies of defined specificity using whole T cell clones as immunogens and selecting for TCR reagents of defined isotype, can readily be extended to producing additional anti-minor framework monoclonal antibodies. Antibodies to Vβ12.1, Vfβ1.2, Vβ12.4, Vβ2, Vβ9, and Vβ10 are being generated by immunizing with the T cell clones or cell lines listed in Table 4. $\alpha\beta^+$ T cell clones can likewise be used to produce anti-minor framework or viable region specific anti-γ and anti-δ reagents.

TABLE 4

| Cell line | Vβ | Vα | Dβ | Jβ |
|---|---|---|---|---|
| C11 | 2.? | | | |
| M11 | 2.2 | | 2.1 | 2.3 |
| DT259 | 3.2 | | 1.1 | 2.1 |
| 2G2 | 4.1 | | 2.1 | 2.5 |
| DT110 | 4.2 | | 1.1 | 1.1 |
| HPB | 6.1 | 12.1 | 2.1 | 2.5 |
| 3A1, 5A5 | 6.1 | | | |
| ATL122 | 6.3 | 1.1 | 1.5 | |
| L17 | 6.9 | 17.1 | 1.1 | 1.5 |
| Jurkat | 8.1 | 1.2 | 1.1 | 1.2 |
| CEM2 | 9.1 | | | 2.3 |
| ATL121 | 10.2 | | 1.1 | 1.5 |
| CEM1 | 12.4 | | | |
| ATL21 | 15.1 | | 1.1 | 1.5 |
| HUT102 | 20.1 | | | 1.2 |
| DT55 | | 4.3 | | |
| SUPT1 | | 7.2 | | |
| HUT 78 | 18.1 | 1. | | |
| MOLT 16 | 1. | 2. | | |

## 8. EXAMPLE: ELEVATION OF A γδ T-CELL SUBSET IN PERIPHERAL BLOOD AND SYNOVIAL FLUID OF PATIENTS WITH RHEUMATOID ARTHRITIS

The levels of δTCS1 positive and TCRδ1 positive T cells were determined in the peripheral blood and synovial fluid of patients with rheumatoid arthritis (RA) and Felty's syndrome (FS). Elevated levels of a γδ T cell subset identified by δTCS1 was found in the peripheral blood and synovial fluid of patients with RA as well as in the peripheral blood of patients with FS.

8.1. MATERIALS AND METHODS

8.1.1. PATIENT SELECTION

Twenty-nine patients with definite or classical RA and 11 additional patients with Felty's Syndrome seen in the Rheumatic Disease Unit of the Wellesley Hospital were studied. Controls consisted of 22 healthy volunteers (NML) from the Wellesley Hospital and T Cell Sciences, Inc. Twelve of the controls were age-matched. Since the data obtained from the age-matched control subjects from the two groups were comparable to those not matched for age, all control data was pooled. In addition, 5 patients with seronegative spondyloarthropathies (3 with psoriasis, 1 with Reiter's syndrome and 1 with ankylosing spondylitis) were examined. All patients were receiving nonsteroidal anti-inflammatory drugs (NSAIDs). In addition to NSAIDs, some of the RA patients had received at various times during active disease stages any of the following: remittive agents, prednisone, or cytotoxic agents/remittive agents.

8.1.2. CLINICAL EVALUATION

A number of clinical variables were examined in each patient, including: 1) disease duration, 2) number of actively inflamed joints (defined as those with tenderness or effusions), 3) erythrocyte sedimentation rate (ESR; Westergren) and 4) medications.

8.1.3. PREPARATION OF MONONUCLEAR CELLS FROM PERIPHERAL BLOOD

Equal volumes of whole blood (containing anti-coagulant) and Sepracell-MN (Sepratech Corp.) were added to a centrifuge tube. After gentle mixing, the tube was centrifuged at 1500×g at room temperature for 20 minutes. After density separation, mononuclear cells found in the opalescent compact band just below the meniscus were collected. They were washed twice by mixing the cells with four volumes of PBS-BSA (0.1% w/v), and subjecting the mixture to centrifugation at 300×g for 10 minutes per wash.

8.1.4. IMMUNOFLUORESCENCE STAINING OF CELL SURFACE MARKERS

All patient cells were phenotyped by flow cytometry using the Ortho Diagnostics Cytofluorograph II (Ortho Diagnostic Systems, Inc., Raritan, N.J.). Fluorescein-conjugated monoclonal antibodies specific for various cell surface determinants were used for a direct immunofluorescence staining procedure. Briefly, 2–5×$10^5$ mononuclear cells were suspended in 100 μl of PBS with 0.2% bovine serum albumin and 0.05% sodium azide (flow buffer) at 4° C. Conjugated monoclonal antibodies (100 ng) were added to the cell suspension mixed well and incubated for 30 minutes at 4° C. The stained cells were washed with flow buffer two times and finally resuspended in the same buffer for cytometric analysis. The monoclonal antibodies utilized for immunofluorescence included the following from Ortho Diagnostics Systems, Inc. (Raritan, N.J.), OKT3, OKT4, and OKT8, which recognize the CD3, CD4 and CD8 determinants, respectively, and TCRδ1 and δTCS1, which recognize all human γδ T cells and a subset of them, respectively.

8.2. RESULTS

We first examined the levels of TCRδ1$^+$ T cells and δTCS1$^+$ cells in the peripheral blood of patients with rheumatoid arthritis (RA) and compared them to the levels in peripheral blood of patients with Felty's synodrome (FS) and healthy control subjects (NML). The results (FIG. **11**, left panel) revealed comparable log mean levels of peripheral blood TCRδ1$^+$ T cells in RA (5.2%) and NML (3.9%) patients (p>0.05), whereas FS patients exhibited higher levels (6.8%) relative to the other groups; however, the difference was not statistically significant (p>0.05). In contrast, mean levels of PB δTCS1$^+$ T cells in RA (1.8%) and FS (4.5%) were significantly elevated relative to NML (0.8%) (p=0.007 and p=0.006, respectively) (FIG. **11**, right panel). The level of δTCS1$^+$ T cells in RA was not statistically different from that in FS (p>0.05).

The level of peripheral blood δTCS1$^+$ T cells was next examined as a proportion of TCRδ1$^+$ cells. The results (FIG. **12**) revealed an elevation in the mean ratio of δTCS1/TCRδ1 in both RA (0.4) and FS (0.6) relative to NML (0.2) (p=0.02 and p=0.005, respectively). Moreover, in patients with FS who demonstrated elevated levels of TCRδ1$^+$ T cells, δTCS1$^+$ T cells accounted for the majority of TCRδ1$^+$ T cells while in normals, δTCS1 T cells account for <20% of the TCRδ1$^+$ T cell population. These results suggested a correlation between the level of δTCS1$^+$ T cells and TCRδ1$^+$ T cells. Indeed, the level of δTCS1$^+$ T cells correlated strongly with the level of TCRδ1$^+$ T cells in FS (r=0.98, p=0.001) (FIG. **13**).

Since elevated levels of peripheral blood δTCS1$^+$ T cells were observed in RA, we next asked whether the increased peripheral blood levels could be a reflection of a local increase in δTCS1$^+$ T cells within the synovium. To address this issue, we evaluated the levels of δTCS1$^+$ T cells and TCRδ1$^+$ T cells in paired synovial fluid and peripheral blood samples from 16 RA patients and compared these levels to synovial fluid samples from 9 patients with seronegative spondyloarthropathies (SSA), five of whom also had peripheral blood levels determined. The results (FIG. **14**, lower panel) revealed comparable log mean levels of δTCS1$^+$ cells in paired RA peripheral blood and synovial fluid samples (1.9% and 2.2%, respectively). Moreover, the level of δTCS1$^+$ T cells in RA peripheral blood was elevated relative to the peripheral blood level in SSA patients (0.05%) (p=0.05), and there appeared to be a difference between the synovial fluid levels in RA and SSA (p=0.1). The level in RA peripheral blood and RA synovial fluid, but not in SSA synovial fluid, was greater than that in the peripheral blood NML group. The levels of TCRδ1$^+$ T cells in paired RA peripheral blood and synovial fluid samples were comparable (5.2% vs 5.2%) and not significantly different from that in peripheral blood of NML controls (3.6%) (p>0.05 for both comparisons (FIG. **14**, upper panel). Again, there was a trend toward a lower synovial fluid level in SSA (3.0%) relative to RA peripheral blood and synovial fluid levels.

When we examined δTCS1/TCRδ1 ratios in paired peripheral blood and synovial fluid samples, we observed comparable ratios in RA peripheral blood (0.40), RA synovial fluid (0.5) and SSA synovial fluid (0.6), (p>0.05 for all comparisons) (FIG. **15**). However, the δTCS1/TCRδ1 ratios in RA synovial fluid were significantly elevated relative to that in NML peripheral blood (0.2±0.5) (p=0.005). Of note, the δTCS1/TCRδ1 ratio in SSA synovial fluid was also elevated relative to NML peripheral blood (p=0.04). The ratio in SSA peripheral blood was comparable to those in NML peripheral blood and RA peripheral blood (p>0.05 for both).

8.3. DISCUSSION

The results of the present study demonstrate elevated levels of a γδ T cell subset in the peripheral blood and synovial fluid of RA patients and in peripheral blood of patients with FS. In contrast to the preliminary data of Brennan, F. M., et al. (1988, J. Autoimmunity, 1, 319–326) we did not observe an elevation in total γδT cells in the synovial fluid of RA patients. Brennan et al., however, examined paired peripheral blood and synovial fluid samples from only 3 patients.

The pathogenic significance of an elevation in a γδ T cell subset in RA remains unclear. However, an increase in a T cell population in RA synovial fluid with cytotoxic potential is consistent with the observation of spontaneous cytotoxicity of synovial T cells directed against both self and non-self epitopes in RA (Example 9, infra). These findings may not reflect non-specific recruitment of T cells into the inflammatory process; an elevation of a specific subset of γδ T cells was observed, not an elevation in the whole γδ population. Moreover, the elevation of δTCS1$^+$ T cells in RA synovial fluid and not SSA synovial fluid suggests that the increase in δTCS1$^+$ T cells in RA is not a result of non-specific effects of inflammation.

To date, a total patient population of 20 normals, 16 RAs, and 10 Felty's patients has been evaluated for the presence of an elevated δTCS1$^+$ subset in peripheral blood. An elevated level, defined as the mean value observed for the patients plus 2 standard deviations, was observed in 1/20 normals (5%), 4/16 RAs (25%) and 5/10 Felty's (50%). The proportion of patients with elevated δTCS1$^+$ cells in the FS group and RA population was statistically greater than that in the group of healthy subjects with $X^2$=8.9 (p=0.003) and 2.9 (p=0.08), respectively. It is expected that as the general patient population is further divided into subgroups based upon HLA expression and disease state, that these levels of correlation between δTCS1$^+$ cells and disease will increase.

## 9. EXAMPLE: NOVEL AUTOREACTIVE CYTOTOXIC ACTIVITY AGAINST SYNOVIOCYTES BY RHEUMATOID ARTHRITIS DERIVED T CELL LINES AND CLONES

T cell lines were established from synovial tissue and peripheral blood of RA and non-RA patients. Phenotypic

and functional studies indicated that specific T cell recruitment occurs in reheumatoid arthritis joints.

9.1. MATERIALS AND METHODS

9.1.1. PATIENT SAMPLES

A total of 23 paired RA patient synovial tissue and peripheral blood samples were collected for this study. All the patients had seropositive classic RA with ages ranging from 38 to 77 years with a mean age of 59.5. In addition to the RA patient samples, we also obtained tissue samples from 8 non-RA patients. Seven of these patients had traumatic arthritis of the knee (i.e., torn meniscus) and their synovial tissue was derived by arthroscopy. One of the eight (NST-7) non-RA tissue specimens was from the knee of a patient with ankylosing spondilitis. In the non-RA patient group, a peripheral blood sample was obtained only from the ankylosing spondylitis patient.

9.1.2. SAMPLE PROCESSING AND CELL LINES

A panel of various cell lines were derived from each patient, whenever possible. T cell lines were obtained from both the disease site and the peripheral blood.

9.1.3. SYNOVIAL TISSUE-DERIVED T CELL LINES

Synovial tissue-derived T cell lines from RA patients (ST-line) were initially established by placing finely minced synovium in a 24 well plate (Costar, Cambridge, Mass.) containing IL-2 medium which consisted of RPMI 1640 supplemented with 10% fetal calf serum (Hyclone, Logan, Utah.), 100 Units/ml penicillin, 100 ug/ml streptomycin, 1 mM sodium pyruvate, 50 ng/ml gentamycin, 10 mM Hepes and 20 Units/ml of recombinant interleukin-2 (Il-2) (Amgen, Thousand Oaks, Calif.). After the culture conditions had been optimized, the minced synovium was instead placed into fetal calf serum free medium consisting of AIM-V (Gibco, Grand Island, N.Y.), 5% human AB serum (Center for Diagnostic Products, Milford, Mass.), 5% LymphocultT (Biotest, Fairfield, N.J.) and 20 u/ml recombinant IL-2. The activated T cells within the diseased synovium migrated out of the tissue in the presence of IL-2 medium. The ST-line T cells were maintained by replacing half of the culture volume with fresh IL-2 medium twice a week. After 3 weeks in culture, the T cells required additional stimulation. Initial cultures were stimulated with allogeneic, irradiated feeder cells from normal peripheral blood lymphocytes (PBL) at a concentration of $2\times10^5$/well and 100 ng/ml of a CD3-specific monoclonal antibody, OKT3. ST-line T cells were cloned by limiting dilution in round bottom 96 well plates (Costar, Cambridge, Mass.) with $1\times10^5$ feeder cells per well. After optimization, later cultures were stimulated by growing the T cells on CD3 coated 24 well plates (coated with 2.5 $\mu$g/ml OKT3) for a minimum of 3 hours, followed by washing 3 times and then plating the cells. It was observed that the use of allogeneic and even autologous feeder cells biased the cell growth toward $CD8^+$ cells during culture. The lines maintained their original phenotype better on CD3 coated plates.

9.1.4. PERIPHERAL BLOOD-DERIVED T CELL LINES

Peripheral blood-derived T cell lines (PB-T) were obtained by culturing PBL in IL-2 medium plus 1 ug/ml PHA (Welcome Research Laboratories, Beckenham, England).

9.1.5. B CELL LINES

B cell lines were derived from PBL by Epstein-Barr virus (EBV) transformation (Alpert, S. D., et al., 1987, J. Immunol., 138, 104).

9.1.6. PERIPHERAL BLOOD MACROPHAGES

Peripheral blood macrophages (PBMO) were obtained by overnight adherance of PBL in RPMI medium plus 10% human AB serum (Center for Diagnostic Products, Milford, Mass.). The non-adherant cells were thoroughly washed away and the adherant macrophages isolated by incubation with ice cold calcium/magnesium free phosphate buffered saline (PBS).

9.1.7. SYNOVIOCYTES

Synoviocytes were also obtained by culturing in IL-2 medium in either 100mm dishes in which the tissue was minced or in 6 well dishes (Costar, Cambridge, Mass.). The medium was usually supplemented with human serum unless otherwise stated. Type B synoviocytes were fibroblast cells which were negative for nonspecific esterase (Sigma, St. Louis, Mo.) and HLA-DR while Type A synoviocytes were nonspecific esterase and HLA-DR positive (Carson, D. A. and Fox, R. I., 1985, In "Arthritis and Allied Conditions", McCarty, D. J. (Ed.), p. 257; Iguchi, T., et al., 1986, Arth. Rheum., 29, 600). Separation of Type A and Type B cells was accomplished by differential adherance and trypsin sensitivity. Synovial fibroblast cultures could be obtained by 3–5 minute trypsinization from a tissue monolayer outgrowth or by allowing the fibroblasts to overgrow the primary mixed culture. The mixed cultures that had 3–5 minute trypsin treatment resulted in greater than 95% pure Type A, macrophage-like cultures, as measured by esterase staining. Not all patient samples generated Type A cells in adequate numbers for functional assays. Both types of synoviocytes, PB macrophages and the EBV-B cell lines were used as autologous targets in cytotoxicity assays.

9.1.8. CELL SURFACE PHENOTYPING

All patient cell lines were phenotyped by flow cytometry using the Ortho Diagnostics Cytofluorograf II. Fluorescein-conjugated monoclonal antibodies specific for various cell surface determinants were used for a direct immunofluorescence staining procedure. Briefly, $2–5\times10^5$ cells were suspended in 100 $\mu$l of PBS with 0.2% bovine serum albumin and 0.1% sodium azide (flow buffer) at 4° C. Conjugated monoclonal antibodies (100 ng) were added to the cell suspension mixed well and incubated for 30 minutes at 4° C. The stained cells were washed with flow buffer two times and finally resuspended in the same buffer for cytometric analysis. The monoclonal antibodies utilized for T cell phenotyping include the following from Ortho, Raritan, N.J.; OKT3, OKT4, OKT8, which recognize the the CD3, CD4 and CD8 determinants, respectively. In addition to the T4/T8 subsets, we also stained for the helper-inducer and helper-suppressor subsets using phycoerythrin-conjugated monoclonals 4B4 and 2H4 (Coulter, Hialeah, Fla.). Finally, T cells were also stained with a $V_\delta1$ TCβ R specific monoclonal antibody, δTCS-1. Fluorescein-conjugated HLA DR Class II-specific antibody was purchased from Becton Dickinson, Mountain View, Calif.

9.1.9. CYTOTOXICITY ASSAY

T cell mediated cytotoxicity was measured by a 3-hour $^{51}Cr$ release assay as previously described (Snider, M. E., et al., 1986, Transplantation, 42, 171). Briefly, target cells were labeled with 50–100 $\mu$C $^{51}Cr$ for 30 minutes at 37° C. in a shaking water bath. After washing the target cells three times in Hanks buffered saline containing 10% serum, they were resuspended in assay medium which consisted of RPMI plus 10% fetal calf serum and 10 mM Hepes. Assay plates were prepared by the addition of 200 $\mu$l volume of effector T cells adjusted to $6.25\times10^4$ per well (resulting in an Effector:Target ratio of 25:1) or by the addition of 200 $\mu$l of medium or water alone. Radioactive target cells were placed in prepared V-bottom 96 well plates (Costar, Cambridge, Mass.) at $2.5\times10^3$ cells in a 20 $\mu$l volume. The plates were spun at 50×g for 5 minutes and incubated at 37° C. in a humidified,

5% $CO_2$ incubator for 3 hours. The assay was harvested by removing 100 $\mu$l of supernatant from each quadruplicate well and radioactivity release was measured on an LKB gamma counter. Percent specific lysis was calculated using the following formula:

$$\% \text{ Specific Lysis} = \frac{ER - SR}{X - B} \times 100$$

where ER=mean $^{51}Cr$ release in the presence of effector T cells, SR=mean spontaneous $^{51}Cr$ release in media alone, X=mean maximum $^{51}Cr$ release in water and B=machine background. Means were calculated from quadruplicate wells and standard deviations never exceeded 10%.

9.1.10. IN SITU IMMUNOHISTOCHEMISTRY

Synovial tissue in about 2 cm square pieces was snap frozen in liquid nitrogen, coated with OCT media, and stored at −70° C. Frozen sections were cut at 5 $\mu$m on a cryostat, placed on microscope slides, air dried, and fixed in acetone for 5 minutes at room temperature. Sections were rehydrated in PBS and stained with δTCS1 at 10 $\mu$g/ml final concentration for 30 minutes at room temperature. Reactivity with δTCS1 antibody was determined using a commercial immunoperoxidase kit specific for mouse Ig (Ortho, Raritan, N.J.). Cells were counterstained using hematoxylin and examined by light microscopy.

9.1.11. SUPPRESSOR FACTOR ASSAY

Normal peripheral blood lymphocytes were stimulated with either OKT3 or PHA. Suppressor activity was determined by the ability of a γδ+ cell culture supernatant to inhibit the proliferative response of PBLs to OKT3 or PHA. Culture supernatants were generated from RA synovial tissue derived T cell cultures or from the paired RA PBL derived T cell cultures following stimulation on either OKT3 or δTCS1 coated plates. Plates were coated first with 2.5 $\mu$g/ml goat anti-mouse IgG1 (Southern Biotech Assoc., Birmingham, Ala.) followed by the appropriate monoclonal antibody at 2.5 ng/ml for 3 hours at 37° C. Control cultures received either no stimulation or stimulation with irradiated allogeneic peripheral blood lymphocytes plus 100 ng/ml OKT3. Monoclonal antibody coated plates were selected to eliminate the production of lymphokines by the allogeneic feeders. Normal PBLs were plated in 96 well flat bottom plates at $8\times10^4$ cells/well in RPMI 1640 plus 10% FCS with either 10 ng/ml OKT3 or 0.5 ng/ml PHA (Wellcome Diagnostics, Dartford, England). Culture supernatants were added at a starting dilution of 1:4 with serial dilutions up to 1:1024. Assays were pulsed on day 3 with 1.0 $\mu$Ci/well of tritium for 8 hours before harvesting.

9.2. RESULTS

9.2.1. CELL SURFACE PHENOTYPE OF SYNOVIAL TISSUE-DERIVED AND PERIPHERAL BLOOD-DERIVED T CELLS 9.2.1.1. δTCS1 CELL SURFACE PHENOTYPE

The gamma delta T cell antigen receptor specific monoclonal antibody, δTCS1 (Wu, Y-J., et al., 1988, J. Immunol., 141, 1476–1479), was used to quantitate the gamma delta T cell receptor positive T cells infiltrating synovial tissue. Table 5 compares the percentage of δTCS1 positive T cells from RA patient synovium (ST-line) with the peripheral blood T cells of each of the 23 paired RA samples and 8 paired non-RA samples.

TABLE 5

PERCENTAGE OF δTCS1 POSITIVE T CELLS DERIVED FROM ARTHRITIS PATIENT T CELL LINES*

| Patient Sample | Synovial Tissue | Peripheral Blood |
|---|---|---|
| S1-2 | 12 | 5 |
| ST-9 | 7 | 1 |
| ST-17 | 0 | 7 |
| ST-18 | 2 | 1 |
| ST-24 | 2 | 1 |
| S1-25 | 64 | 11 |
| ST-27 | 10 | 5 |
| ST-28 | 18 | 8 |
| ST-29 | 2 | 13 |
| ST-30 | 0 | 0 |
| S1-31 | 2 | 1 |
| ST-32 | 34 | 0 |
| ST-33 | 13 | 0 |
| ST-34 | 1 | 1 |
| ST-35 | 80 | 3 |
| S1-36 | 1 | 2 |
| ST-37 | 7 | 1 |
| ST-38 | 5 | 0 |
| ST-39 | 9 | 3 |
| ST-43 | 8 | 0 |
| S1-48 | 5 | 3 |
| ST-49 | 1 | 5 |
| ST-51 | 3 | 1 |
| x ± SD | 12.4 ± 20 | 3 ± 4 |
| NST-1 | 2 | NA+ |
| NST-2 | 3 | NA |
| NST-5 | 8 | NA |
| NST-7 | 2 | + |
| NST-9 | 2 | NA |
| NST-10 | 9 | NA |
| NST-17 | 5 | NA |
| NST-13 | 1 | NA |
| x ± SD | 4 ± 3 | NA |

*= double immunofluorescence with OKT3 and δTCS1 monoclonal antibodies. Numbers are expressed as percentage δTCS1 positive cells relative to percentage CD3 positive cells, in order to standardize for samples having varying amounts of CD3+ cells.
+NA = not available (no peripheral blood received)

In normal subjects, gamma-delta T cells comprise 1–5% of peripheral blood T cells (Wu, Y-J., et al., supra; Brenner, M., et al., 1986, Nature, 322, 145). Our results show that while the values of percentage of δTCS1 positive T cells derived from peripheral blood derived T cell lines from RA patients were within the normal range, the values for several paired RA synovial tissue lines were above the normal range (Table 5). Indeed, a marked increase in the proportion of gamma-delta T cells was observed in RA synovial tissue lines. The lower half of Table 5 demonstrates that in most cases the proportion of gamma delta cells from non-RA synovial-derived T cells (NST-lines) are not significantly elevated. These paired patients' T cell lines were cultured in parallel, with the mean length in culture of 18.8±8 days, ranging from 11 to 36 days.

Three of the synovial tissue lines were stained by double immunofluorescence with fluorescein-cinjugated OKT8 and phycoerythrin-conjugated δTCS1. We found a significant percentage of gamma delta+ cells were also CD8+, since the percent of double staining cells for ST-25, ST-32 and ST-33 were 47%, 23%, and 5%, respectively. In addition, the majority of the gamma delta+ cells in ST-35 were $CD4^+$ since the T cell line was 94% CD4+ and 80% $\delta TCS1^+$. These findings suggest that at least in some synovial tissue, the gamma delta+ cells are not members of the $CD4^-$ $CD8^-$ cell population.

9.2.1.2. CD4, CD8, 4B4, 2H4 CELL SURFACE PHENOTYPES:

Synovial tissue lines and paired peripheral blood T cell lines from seven RA patients different from those listed in

Table 5 were analyzed for T cell subset information (i.e. CD4/CD8 ratio and suppressor-inducer/helper-inducer cell ratio). These paired RA patient T cell lines were cultured in parallel with the mean length in culture of 13±1.8 days, ranging from 9 to 15 days. The RA patients' lines were also compared with synovial tissue-derived T cells from four non-RA patients (NST-line). The NST-lines were in culture for a mean of 14±3 days, ranging from 10–19 days. Table 6 confirms previous reports (Forre, O., et al., 1982, Scand. J. Immunol., 16, 815) that T cells derived from diseased joints and stimulated with PHA are predominantly CD4+ (mean value of 79%.±5) and 4B4+ (mean value of 91.4%±6) while the T cells from the peripheral blood of the same patients had an equal mixture of CD4 and CD8 populations (mean values for CD4 and CD8 were 43.8%±17 and 48.8%±12, respectively).

TABLE 6

Comparison of Cell Surface Phenotype of Rheumatoid and Non-Rheumatoid T Cell Lines.*

| | Phenotype Marker | | | | |
|---|---|---|---|---|---|
| Sample | CD4 | CD8 | 4B4 | 2H4 | 4B4/2H4 |
| ST-LINE | 79 ± 15 | 13.9 ± 8.6 | 91.4 ± 6 | 3.1 ± 1.8 | 29.5 |
| ST-PBT | 43.8 ± 17 | 48.8 ± 12.4 | 69.9 ± 14.6 | 22.2 ± 17 | 3.1 |
| NST-LINE | 89 ± 6.5 | 6.7 ± 2.4 | 92.4 ± 8.4 | 1.4 ± 0.7 | 66 |

*% positive ± standard deviation

However, this is the first reported evidence that the phenotype of cultured T cells from synovial tissue of RA and non-RA patients are similar since the NST-line CD4 mean value was 89%±6 and the mean 4B4 value was 92.4%±8. Again, the PBT cells from the RA patients had a more normal distribution of helper-inducer and suppressor-inducer cells, as compared to the infiltrated T cells (i.e. the mean values for ST and PBT were 69.9%±15 4B4 and 22.2%±17 2H4). However, the ratio of 4B4/2H4 in the RA patients was distinct from normal subjects regardless of the source of T cells studied (Morimoto, C., et al., 1985, J. Immunol., 134, 1508).

9.2.2. IN SITU IMMUNOHISTOCHEMISTRY

Samples of synovial tissue were analyzed by in situ staining with δTCS1 to determine whether γδ$^+$ cells were in fact present in inflamed RA joints and that their detection was not due to the selective expansion of a minor T cell subset during the cell culture procedure. As can be seen in Table 7, δTCS1$^+$ cells were present in the majority of infiltrated synovial membrane tissue.

TABLE 7

In situ Staining by T cell Specific Monoclonal Antibodies in Untreated Synovial Tissue

| Sample | DR | Infiltrate | CD3 | CD4 | TCRδ1 | δTCS1 |
|---|---|---|---|---|---|---|
| ST-46 | – | + | + | + | – | – |
| ST-47 | – | ++ | ++ | | + | + |
| ST-48 | – | ++ | ++ | | + | +/– |
| ST-49 | – | – | – | | – | – |
| ST-50 | – | – | – | | – | – |
| ST-51 | – | ++ | ++ | ++ | + | + |
| ST-52 | – | ++ | ++ | | + | + |
| NB-02 | 4 | +++ | +++ | | – | – |
| NB-04 | 4 | +++ | +++ | | + | + |

TABLE 7-continued

In situ Staining by T cell Specific Monoclonal Antibodies in Untreated Synovial Tissue

| Sample | DR | Infiltrate | CD3 | CD4 | TCRδ1 | δTCS1 |
|---|---|---|---|---|---|---|
| NB-05 | 4 | +++ | +++ | | +/– | +/– |
| TW-01 | – | +++ | +++ | | + | + |

Thus, δTCS1 cells have been detected in the peripheral blood, synovial fluid and synovial membrane of patients with RA and in the peripheral blood of Felty's patients.

9.2.3. FUNCTIONAL ACTIVITY OF SYNOVIAL TISSUE DERIVED T CELLS

9.2.3.1. CYTOTOXICITY ACTIVITY

Cytotoxicity of T cell lines from RA synovial tissue against their autologous Type A synoviocytes (macrophage-like cells), Type B synoviocytes (fibroblasts) and peripheral blood macrophages was measured in a 3 hour $^{51}$Cr release assay (Table 8]

TABLE 8

Cytotoxicity of RA Synovial Derived T Cell Lines*

| Effector | Target Cell | | |
|---|---|---|---|
| Cell ST-Line: | Type A Synoviocytes | Type B Synoviocytes | Peripheral Blood Macrophages |
| ST-1 | 80 | 9 | ND |
| ST-9 | 50 | 5 | 5 |
| ST-11 | 41 | 8 | ND |
| ST-13 | 50 | 2 | 6 |
| ST-14 | 42 | 4 | 24 |
| ST-15 | 11 | 7 | ND |
| ST-16 | 1 | 5 | ND |
| ST-17 | 61 | 0 | ND |
| ST-22 | 27 | 2 | ND |
| ST-25 | 26 | 6 | 13 |
| ST-28 | 0 | 7 | 4 |

*= % specific lysis in 3 hour $^{51}$Cr release assay.

ST-lines derived from nine out of eleven patients demonstrated cytotoxicity against autologous Type A synovial target cells while not affecting the viability of the Type B synovial cells or PB macophages. In addition, autologous B cells were not lysed by the ST-lines. The natural killer (NK)-like activity of the T cell lines was variable and did riot correspond with the lytic activity against autologous synovial macrophage-like cells. In fact, cloning of T cells from a patient (ST-1) whose line exhibited high NK-like activity resulted in Type A-specific clones with no detectable NK-like activity (Table 9).

TABLE 9

Cytotoxic Activity of Synovial Tissue Derived T Cell Line and Clones*

| Effector Cell | Target Cells | | |
|---|---|---|---|
| ST-Line or Clone: | Type A Synoviocytes | Type B Synoviocytes | K562 |
| Line | 81 | 9 | 63 |
| Clone 1 | 58 | 0 | 8 |
| Clone 2 | 60 | 0 | 1 |

TABLE 9-continued

Cytotoxic Activity of Synovial Tissue Derived T Cell Line and Clones*

| Effector Cell | Target Cells | | |
|---|---|---|---|
| ST-Line or Clone: | Type A Synoviocytes | Type B Synoviocytes | K562 |
| Clone 3 | 32 | 0 | 2 |
| Clone 4 | 24 | 0 | 0 |

*= % specific lysis in 3 hour $^{51}$Cr release assay.

Table 9 also shows that both CD4+ and CD8+ clones could specifically lyse Type A synoviocytes since clones 1, 3 and 4 were CD4+ while clone 2 was CD8+.

The specificity of ST-lines for their syngeneic tissue-derived targets was not shared by the peripheral blood T cell lines from the same patients. In fact, the peripheral blood derived T cells followed two patterns of cell lysis: either they were very lytic against the entire target cell panel including K562, or they had very weak activity against K562 and occasionally other target cells as well. An example of the latter is shown in Table 10.

TABLE 10

Comparison of the Cytotoxic Activity of a Synovial-Derived T Cell Line and a Peripheral Blood T Cell from a Single Patient.

| | % Specific Lysis By:* | |
|---|---|---|
| TARGET CELLS | ST-Line | ST-PBT |
| Type A Synoviocytes | 50 | 3 |
| Type B Synoviocytes | 2 | 4 |
| PB-Macrophage | 6 | 21 |
| EBV-B Cell | 0 | 0 |
| K562 | 11 | 18 |
| Daudi | 2 | 1 |

*The effector-to-target cell ratio was 25:1 and this experiment was done with the RA patient, ST-13.

In this case, one patient's (ST-13) ST-line and PB-T cytotoxicity was measured against a panel of autologous target cells, K562, and the lymphokine-activated killer (LAK)-sensitive target cell, Daudi. The ST-line lysed the Type A cells and had some detectable natural killer cell-like activity while not lysing any other target cells in the panel. However, the PB-T cells from the same patient which were cultured in parallel did not lyse either of the autologous synoviocytes but did have some NK-like activity and lysed some of the PB macrophages. To examine the specificity of ST-13 line activity more closely, a cold target inhibition assay was performed (FIG. **16**). Lysis of $^{51}$Cr labeled Type A cells by ST-line T cells was directly inhibited by the addition of increasing numbers of unlabeled Type A cells. However, addition of increasing numbers of unlabeled K562 did not inhibit $^{51}$Cr release of the Type A cells, suggesting that the lysis of Type A cells is not due to NK-like activity.

The Type A cell specificity of RA synovial tissue-derived T cells was not shared by synovial-derived T cells from the one out of nine non-RA patients(NST-3) where cultures yielded enough Type A synoviocytes for $^{51}$Cr labeling. Table 11 shows the cytotoxic activity of 2 NST-lines and 2 ST-lines against a panel of target cells including autologous synoviocyte targets, allogeneic synoviocyte targets and allogeneic B cells.

TABLE 11

Comparison of the Alloreactivity and Target Specificity by Non-Rheumatoid and Rheumatoid Arthritis-Derived T Cell Lines

| | % Specific Lysis by: | | | |
|---|---|---|---|---|
| Target Cells | NST-3 | NST-7 | ST-17 | ST-22 |
| Autologous Type A cells | 17 | NA | 61 | 27 |
| Autologous Type B cells | 42 | NA | 0 | 2 |
| Allogeneic Type A cells | 0 | 3 | 7 | 34 |
| Allogeneic Type B cells | 7 | 2 | 39 | 4 |
| Allogeneic EBV-B cells | 2 | 0 | 33 | 40 |

The allogeneic synoviocyte and B cell targets were derived from Rheumatoid Arthritis patients.
NA = target cells not available

NST-3 line T cells lysed Type B cells to a greater extent than the Type A cells and neither NST-lines had any significant alloreactivity. In contrast, the synovial tissue derived lines both appeared to lyse the Type A cells while not affecting the Type B cells. In addition, the T cells from these two patients demonstrated alloreactivity against Epstein Barr Virus B cells a ytolytic activity against allogeneic Type A cells for ST-22 or allogeneic Type B cells for ST-17. This alloreactivity was only seen in these 2 patients from the panel of 11 patients tested to date.

9.2.3.2. CYTOTOXIC ACTIVITY OF A γδ POSITIVE T CELL LINE; EFFECT OF δTCS1 MONOCLONAL ANTIBODY

T cell line ST-25, derived from the synovial tissue of a rheumatoid arthritis patient, was assayed for cytotoxic activity against its autologous target cells. The effector cells were pretreated with δTCS1 (2 μg/ml) and then washed. The anti-HLA-DR (Becton Dickinson, Mountain View, Calif.) and the anti-KLH (Keyhole limpet hemocyanin)control antibodies were added at the initiation of the assay (2 ng/ml) and were present during the 3 hour assay. At the time of the assay, the phenotype of ST-25 was 41.8% double positive for δTCS1 and CD3 and 39.3% double positive for δTCS1 and CD8. The results of the ST-25 killing assay are given in Table 12.

TABLE 12

Cytotoxicity Data for T Cell Line ST-25*

| Target Cells | ST-25 alone | ST-25 anti-HLA-DR antibody | ST-25 plus δTCS1 antibody | ST-25 plus anti-KLH antibody |
|---|---|---|---|---|
| ST-25 Type A Synoviocytes | 17 | 7 | 52 | 15 |
| ST-25 Type B Synoviocytes | 9 | ND | ND | ND |
| ST-25 B Cell Line | 0 | ND | ND | ND |
| ST-25 PBMO | 24 | 14 | 124 | 43 |
| PB Line 13 | 0 | 0 | 4 | ND |
| PB Line | 0 | 1 | 21 | 0 |
| K562 | 29 | ND | ND | ND |

*= % specific lysis at an effector target ratio of 25:1
ND = not determined

The ST-25 cell line effectively killed ST-25 autologous Type A synoviocytes and peripheral blood macrophages and K562 cells. Preincubation of ST-25 cell line with an anti-HLA-DR antibody (Becton Dickinson, Mountain View, Conn.) caused a slight inhibition of the assay, while a control antibody to keyhole limpet hemocyanin (KLH) caused little effect. Treatment with δTCS1, however, resulted in a significant enhancement of the lysis.

In summary, the activity of some subsets of γδ positive cells may be cytotoxic and it may be possible to enhance the killing activity of this subset by treatment with δTCS1 monoclonal antibody. This antibody also shows mitogenic activity (Wu, Y-J., et al., 1988, J. Immunol., 141, 1476–1479). Using appropriate amounts of antibody, δTCS1 binds to the surface of γδ positive cells and stimulates the proliferation of a minor cell population of resting human PBL to levels where the proportion of γδ positive cells in the culture exceeds 90%.

9.2.3.3. SUPPRESSOR ACTIVITY OF γδ POSITIVE ST CELL LINES

For the suppression assays, fresh peripheral blood lymphocytes were prepared and then stimulated with either PHA or anti-CD3 monoclonal antibody. The inhibition of the proliferation of these cells was tested by adding varying dilutions of supernatant fluid obtained from the cell cultures of ST-25 and ST-32 cell lines. Supernatant fluid from both ST-25 and ST-32 cell lines suppressed the proliferation of PHA or anti-CD3 stimulated PBLs. This suppression was observed at supernatant dilutions exceeding 1:1024 for ST-32 and 1:256 for ST-25. The factors are soluble and are not removed by dialysis overnight at 4° C. (molecular weight cutoff of 3,000). The antibody, δTCS1, is mitogenic for γδ cells (see 9.3.3.2, supra) and it may be that, following the triggering of these cells with the antibody, suppressor factors are produced. This would result in the ability to modulate this affect in the $\gamma\delta^+$ T cell subset by using the appropriate dosage of antibody.

9.2.4. BLOCKING OF THE CYTOTOXICITY REACTION BY δTCS1

To establish the ability of δTCS1 monoclonal antibody to block the cytotoxic activity of $\delta TCS1^+$ effector cells against specific and non-specific target cells, the RA derived effector cells may be preincubated with δTCS1 antibody using a range of concentrations. To distinguish between Fc receptor dependent killing and δTCS1 specific blocking in the cytotoxicity assays, both whole antibody and Fab fragments of δTCS1 antibody may be tested. The whole antibody may be used to give rise to Fc receptor mediated killing, whereas the Fab fragment (which has the Fc portion of the antibody removed) may be used to effect specific blocking of $\gamma\delta^+$ effector cell cytotoxic activity.

9.2.5. DEPLETION ASSAY USING δTCS1

To establish the ability of δTCS1 monoclonal antibody to modulate $\gamma\delta^+$ T cells and cause their specific elmination in vitro, δTCS1 may be tested in complement mediated lysis assays. For these assays, RA derived effector cells may be preincubated with δTCS1 antibody using a range of concentrations. Following preincubation, rabbit serum may be added as a source of complement pathway components. With the appropriate concentrations of δTCS1, the complement cascade may become activated leading to complement dependent lysis of the $\delta TCS1^+$ cells and their depletion from the cell population. Effective in vivo concentrations of δTCS1 for T cell elimination may be estimated to be approximately equal to those observed for the therapeutic monoclonal antibody, OKT3 (see Example 10, infra).

9.3. DISCUSSION

The results of this study provide phenotypic and functional evidence that specific T cell recruitment occurs in rheumatoid arthritis joints. It is well known that CD4+ T cells infiltrate the diseased synovium and are in close contact with HLA DR+ synoviocytes (Iguchi, T., et al., 1986, Arth. Rheum., 29, 600; Harris, E. D., Amer. J. Med., 80, 4). This close association of T cells and HLA DR+ tissue macrophage-like cells is thought to be responsible for propagating the inflammatory response in the synovium of RA patients. The exact nature, or even the existence of specific antigens causing RA is unclear. However, it is believed that antigens may be presented by the synoviocytes with elevated HLA DR, the appropriate class II restricting element for CD4+ cells. Therefore, we isolated and expanded the diseased tissue-infiltrated T cells for phenotypic and functional characterization with a specific focus on the interaction of those T cells and the other cell types found in the diseased, autologous synovium.

A novel functional response from synovial tissue-derived T cells was observed. Short-term cultured lines and cloned T cells from diseased joints were able to specifically lyse autologous Type A macrophage-like synoviocytes, in vitro. Previous studies have shown that peripheral blood derived T cells could lyse synovial type B fibroblasts, however, this was observed primarily against allogeneic fibroblasts (Griffiths, M. M., et al., 1976, J. Clin. Invest., 58, 613; Person, D. A., et al., 1876, J. Clin. Invest., 58, 690). Our data represents the first report of autologous Type A synoviocyte cytotoxicity by synovial tissue derived T cells, and the lytic activity we observed in the PB-T lines was mostly of an NK-like nature. The structure(s) recognized by the ST-lines and clones are not known; however, it does not appear to be class II antigen alone since DR+ autologous peripheral blood macrophages and B cells were not killed in the same assays. Attempts are ongoing to isolate adequate numbers of Type A cells for future molecular analysis of unique proteins. In the meantime, T cells exhibiting this unique pattern of target cell recognition are abundant.

Cell surface staining with a monoclonal antibody which recognized the second type of T cell antigen receptor, γδ cells, showed that RA synovium contained significantly higher numbers of $\gamma\delta^+$ T cells than those found in peripheral blood of the same patient. In addition, those gamma delta+ cells were not from a double negative CD4-CD8-population since they stained brightly for either CD8 or CD4 in those patients studied by double immunofluorescence. The selection of γδ+ T cells seen in RA synovium but not in non-RA synovium has not been previously reported. The specificity and function of human γδ T cells is not yet fully understood; however, preliminary reports suggest that they may be HLA-unrestricted killer cells (Faure, F., et al., 1988, J. Immunol., 140, 1372) and murine γδ cells are thought to be alloreactive (Maeda, K., et al., 1987, Proc. Natl. Acad. Sci. USA, 84, 6536; Matis, L. A., et al., 1987, Nature, 330, 262). Preliminary information on the cytotoxicity of the RA-derived γδ+ T cell lines indicates some direct alloreactivity in ST-28 line while no detectable alloreactivity was observed with the others tested.

The synovial tissue of RA patients is known to be infiltrated with the helper-inducer subset of T cells by immunofluorescence of frozen membrane sections (Duke, O., et al., 1987, Arth. Rheum., 30, 849). We have confirmed this observation using isolated T cells obtained from pieces of synovium cultured in the presence of IL-2 medium. There is some discrepancy concerning the ratio of CD4+ to CD8+ cells in the peripheral blood of RA patients. Some reports indicate an increased number of CD4+ cells (Fox, R. I., et al., 1982, J. Immunol., 128, 351), others show a decrease in the CD4+ cells (Forre, O., et al., 1982, Scand. J. Immunol., 15, 221) and still others observe no change in the T cell ratios in peripheral blood (Silverman, H. A., et al., 1976 Arth. Rheum., 19, 509). Our data demonstrates a decreased relative number of CD4+ cells and an increased number of CD8+ cells as compared to the values found in PHA stimulated peripheral blood, resulting in a lower than normal

ratio of CD4/CD8 cells. In addition, we found a lower than normal level of 2H4+ cells in the peripheral blood of RA patients; however, the defect in suppressor-inducer cells is more dramatic in the tissue than in the blood. Moreover, the major T cells involved in the pathogenesis of joint damage are the helper-inducer T cells and the normal counterbalance provided by the suppressor-inducer cells was lacking in the joint and reduced in the periphery of RA patients.

Several reports have indicated that the T cell abnormalities in RA include diminished suppressor cell activity, decreased mitogenic responses and increased antibody production (Silverman, H. Aa., et al., 1976, Arth. Rheuem., 19, 509; Indiveri, F., et al., 1986, Cell. Immunol., 97, 197; Wernick, R. Mm., et al., 1985, Aarth, Rheum., 28, 742). Also, depressed lymphokine production and responsiveness have been reported for both IL-2 and gamma-interferon (Lotz, M., et al., 1986, J. Immunol., 136, 3643; Husby, B. and Williams, R. C., 1985, Arth. Rheum., 28, 174). In most cases, however, T cell responses were not measured in the context of synovial tissue-derived antigens. Our data demonstrated the effector function against autologous synovial tissue-derived antigens. The cytotoxicity does not appear to be mediated by NK-like mechanisms. Evidence for this tissue specificity in vivo is yet to be determined but the implication is that the infiltrating T cells may be directly contributing to the membrane damage in the disease process. In fact, the quantitative relationship between Type A and Type B synoviocytes in the synovial membrane is known to be altered in RA (Carson, D. A. and Fox, R. I., 1985, In "Arthritis and Allied Conditions", McCarty, D. J. (Ed.), p. 257). An increased number of Type A cells early in the synovitis may even provide a mechanism of selection in directed homing of T cells capable of recognizing Type A antigen(s). This hypothesis of T cell selection in the diseased joint is supported by the finding that T cells in the PB of RA patients did not exhibit the same pattern of target specificity as T cells cloned directly from the synovium. Additional evidence for a disease-related T cell migration into the synovium may include the lack of Type A specificity in the T cells derived from non-RA synovium, but more patient samples must first be tested.

In summary, we provide evidence of a novel functional specificity mediated by RA synovial-derived T cells. T cells from RA peripheral blood or non-RA synovium did not show any preferential target cell lysis which indicated that the unique structures) recognized by synovial tissue lines may be disease related. Many of the synovial tissue lines exhibited a significantly higher percentage of γδ+ T cell antigen receptors as compared to either peripheral blood or non-RA synovial-derived T cells. In contrast, the CD4/CD8 ratios and 4B4/2H4 ratios were similar in ST-lines and NST-lines implying no disease correlation with these classes of T cell subsets infiltrating the synovial membrane. Because of this, the nature of the TCAR specificity should be more informative than studying the surface CD4, CD8, 4B4, 2H4 or similar surface phenotypes of T cell subsets. Most data to date support the hypothesis that RA patients, as well as other autoimmune patients (Maeda, K., et al., 1987, Proc. Natl. Acad. Sci. USA, 84, 6536), lack sufficient suppressor cells to control autoreactivity. Our findings are consistent with an aggressive, but novel, autoreactivity and depressed suppressor inducer T cells in RA patients. Studies are underway to study the molecular nature of the T cell antigen receptors found in the diseased synovium (see Example 8, surra and Example 11, infra) and should provide information on the autoimmune mechanisms propagating chronic RA. The contribution of T cell-mediated Type A synoviocyte destruction in RA or other inflammatory arthrophathies remains unclear and is being studied further.

## 10. EXAMPLE: δTCS1, A MONOCLONAL ANTIBODY REACTIVE WITH THE Vδ1 REGION OF HUMAN T CELL ANTIGEN RECEPTOR, IS USEFUL IN THE TREATMENT OF RHEUMATOID ARTHRITIS

Our results show that under different conditions, the antibody δTCS1 can specifically modulate the activity of a subset of γδ+ T cells. These cells are elevated in the synovial fluid and peripheral blood of RA patients and in the peripheral blood of Felty's patients. In addition, they are present in the population of T cells that infiltrates the inflamed synovial tissue of RA patients. Improved therapies can be developed using δTCS1, because this antibody can selectively modulate a specific disease related subset of T cells and not affect other T cell populations. Depending upon the dosage of antibody used, δTCS1 based immunotherapies will either stimulate the specific T cell subset or casue it's elimination from the body; and enhance or block the T cell subset's function. For anti-CD3 monoclonal antibody treatments, different elimination and stimulation therapies have been described (Chatenoud et al., 1982, Eur. J. Immunol. 12:979–982; Hirsch et al., 1989, J. Immunol. 142:737–743; Chatenoud et al., 1988, C. R. Acad. Sci. Paris, 307:833–836). The protocol given below as an example is one designed to specifically deplete δTCS1$^+$ T cells from patients. in whom their presence is deleterious.

### 10.1. PRECLINICAL DATA

#### 10.1.1. PERIPHERAL BLOOD STUDY IN RA AND FELTY'S PATIENTS

During a preliminary study of the T cell subsets in RA patients in 1987, we observed a patient with extremely elevated δTCS1$^+$ T cells (70%) in his blood. Upon further inquiry, the patient was diagnosed to have Felty's Syndrome. To date, our study of γδ+ T cells in disease has included 40 RA patients, 11 Felty's patients, 5 non-RA joint disease patients and 22 age matched normals. As shown in Example 8, 45.5% of the Felty's and 27.5% of the RA patients showed significant elevations of δTCS1$^+$ T cells (mean of normal plus 2 standard deviations) in peripheral blood.

The prevalence of various autoimmune diseases is associated with well defined HLA phenotypes (see Table 13).

TABLE 13

Some HLA-DR-Associated Autoimmune Diseases

| Disease | HLA Type | PATIENT % | NORMAL % | Relative Risk |
|---|---|---|---|---|
| Felty's Syndrome | DR4 | 95 | 20 | 76 |
| RA with Vasculitis | DR4 | 95 | 20 | 76 |
| RA | DR4 | 68 | 25 | 3.8 |
| IDDM | DR4 | 72 | 24 | 9.1 |
| | DR3 | 49 | 22 | 4.3 |

RA = Rheumatoid Arthritis
IDDM = Insulin dependent diabetes mellitus

If Felty's Disease patients or RA patients are further divided into sub-groups based upon their HLA DR and Dw phenotypes, it is probable that the elevation of δTCS1$^+$ T cells will correlate even more strongly with disease.

#### 10.1.2. LONGITUDINAL STUDIES:

It is expected that the elevation of δTCS1$^+$ T cells in RA or Felty's will correlate with disease state (e.g., tender joints,

vasculitis, etc.). In addition, it is well known that female RA patients undergo remission of symptoms shortly after becoming pregnant. Their symptoms return again after giving birth. Longitudinal studies are underway to study the levels of δTCS1$^+$ T cells in RA and Felty's patients and in pregnant women. These numbers may then be correlated with the intensity of the disease during these stages. This data may be used to subdivide patients that would benefit from δTCS1 therapies designed to modulate (elimate or block or stimulate or enhance) the δTCS1$^+$ T cell subset.

10.1.3. SYNOVIAL FLUID AND SYNOVIUM TISSUE STUDY

Analysis of RA synovium-derived T cells upon expansion in IL2 supplemented culture fluid and of synovial fluid T cells yielded elevated levels of δTCS1$^+$ cells in RA patients as was seen for peripheral blood (see Examples 8 & 9, supra). In many RA samples, paired blood and synovium tissues from the same patients were studied (see Example 9, supra). Furthermore, we further evaluated the δTCS1$^+$ T cells in RA synovium by using in situ immunohistochemistry (Example 8, supra). 6 out of 11 RA patients studied showed significant infiltration of δTCS1$^+$ T cells in synovium. This indicated that the elevation of δTCS1$^+$ was not due to a bias in cell growth during in vitro culture conditions. The detailed relationship between the δTCS1$^+$ T cells in the blood and synovium of the same RA patient is being investigated further.

10.1.4. ACTIVITY OF Δδ$^+$ T CELLS IN RHEUMATOID ARTHRITIS

The γδ$^+$ cells from some RA synovial tissue derived T cell lines possessed cytotoxic activity against their autologous type A synoviocytes, and other lines produced factors with suppressor activity. The antibody, δTCS1, was able, under varied conditions, to either enhance the cytotoxic activity, to mitogenically stimulate the cells, or to block the activity of the cells (Example 9, supra).

Taken together the data suggests that δTCS1$^+$ T cells play a significant role in the pathogenesis of rheumatoid arthritis patients and that it can be used as a T cell receptor-specific therapeutic.

10.1.5. αβ T CELL ANALYSIS IN ARTHRITIS

Evidence has been generated that T cells infiltrating synovium of some RA patients primarily express Vβ3, Vβ9, and Vβ10 (Example 11, infra). These 3 Vβ's together account for 5% of total T cells in normal subjects. This demonstrated that distinct subsets of T cells that represent only a small fraction of the T cells in normal subjects may be preferentially associated with some RA patients. Thus, it appears that at least two distinct groups of RA patients can be determined based upon TCAR expression; the first group expresses Vβ3, Vβ9, or Vβ10 in the synovium, while the second group expresses primarily Vδ1.

10.1.6. TOXICOLOGY OF δTCS1 MONOCLONAL ANTIBODY

The acute and chronic toxicity of δTCS1 antibody may be determined by standard animal model procedures. However, data has been collected with other similar antibodies; e.g. OKT3. The documented information supports the general safety of mouse $IgG_{2a}$ antibody administered to human subjects in amounts up to several grams per person per day (ORTHOCLONE OKT3 (NUROMONAB-CD3) Product Insert, Ortho Pharmaceutical Corp., Raritan, N.J.).

The question of possible cross reactivity of δTCS1 antibody with other human tissues has been exmained. It does not appear to react with any of the other blood cell types, such as neutrophils, monocytes and red blood cells, nor with tissues in the gastrointestinal tracts. It does, however, weakly react with some Langerhans cells in the skin and some intracellular antigen in the glandular cells of endometrium.

Sensitization to a murine monoclonal antibody, such as OKT3, has been observed in most patients under treatment, but has not produced significant symptoms of hypersensitivity, anaphylaxis or serum sickness (ORHTOCLONE OKT3 (MUROMONAB-CD3) Product insert, Ortho Pharmaceutical Corp., Raritan, N.J.). Similar results may be expected for δTCS1.

Almost all of the patients treated with OKT3 developed an acute symptom complex with chills and fever after the first injection. This typically commenced 45–60 minutes after the antibody injection and lasted for several hours. This acute symptom was presumed to be due to a physiological response to the rapid lysis of large numbers of T cells during the therapy (ORTHOCLONE (MUROMONAB-CD3 Product insert, supra). Since δTCS1 antibody will lyse a significantly smaller number of T cells (about 1%), the severity of the side effects associated with OKT3 should be minimized.

10.1.7. PHARMACOKINETICS OF δTCS1 MONOCLONAL ANTIBODY

Data on the pharmacokinetics or pharmacology of δTCS1 antibody may be determined by standard animal model techniques. However, data on this important information has been collected with other mouse monoclonal antibodies, such as OKT3, which may be applicable to δTCS1 antibody.

With a single bolus I.V. injection of 5 mg of OKT3 in a normal subject, the average half life of the antibody averages about 4 hours. During treatment with 5 mg per day for 14 days, mean serum levels of the drug rose increased the first three days and then averaged 0.9 μg/ml on days 3 to 14 (ORTHOCLONE (MUROMONAB-CD3) Product Insert, supra).

10.1.8. PHYSICAL BIOCHEMICAL PROPERTIES OF δTCS1 MONOCLONAL ANTIBODY

The δTCS1 monclonal antibody was generated by fusion of myeloma cells with splenocytes derived from a BALB/c mouse immunized with a human leukemic cell line, Molt 13. Detailed screening, cloning selection, and characterization procedures have been published (Wu, Y-J., et al., 1988, J. Immunol., 141, 1476–1479) and are incorporated by reference herein.

The antibody was initially produced as an IgG1 isotype. To improve the antibody dependent cytotoxicity (ADCC), which is the key in vivo mechanism for cell elimination, an $IgG_{2a}$ variant was selected as described in Section 5.2, supra.

The parent IgG1 isotype and isotype switched $IgG_{2a}$ antibodies were tested by Ouchterloney diffusion, ELISA isotype test, and competition assay. $F(ab)_2$ fragments were also generated and characterized by polyacrylamide gel electrophoresis.

The physiochemical properties of the variant are identical to that of the IgG1 parent in terms of mitogenic properties, cell reactivities and binding. The $IgG_{2a}$ δTCS1 antibody will be used as the therapeutic drug for an elimination protocol.

10.2. CLINICAL PLAN FOR ELIMINATION PROTOCOL

Since one of the therapeutically important properties of δTCS1 monoclonal antibody may be to target the specific δTCS1$^+$ T cell subset for elimination, the following plan is given as a example of this type of protocol.

10.2.1. INDICATION

The intended indication will be for use as adjunctive therapy for treating RA patients who fail or are contraindicated for conventional second line drugs.

10.2.2. PATIENT ADMISSION CRITERIA TO THE STUDY

RA patients who may be enrolled in the study include those who A) exhibit 5% or greater of δTCS1$^+$ T cells in the periperal blood, and B) failed the conventional second line drugs. The screening will be based on the immunofluorescense staining of potential patients' lymphocytes with δTCS1 monoclonal antibody. It is estimated that at least 10 out of 50 severe RA patients screened may be suitable for the study.

10.2.3. CLINICAL ENDPOINT

The initial clinical endpoint relates to a substantial reduction of δTCS1$^+$ T cells and possibly other T cell subsets in circulation within 2 hours after the drug's administration. This will be carried out by the immunofluorescence procedure described above.

The long-term clinical endpoint may be evaluated by clinically monitoring patients and lab indices approved by the American Rheumatism Association (FIG. **17**). The first signs of clinical improvements may come from the reduction of tender joint counts and duration of morning stiffness.

10.2.4. CALCULATION OF DRUG DOSE

A similar approach used for the calculation of OKT3 dose may be employed. Assuming 1) there are 7 liters of blood in an adult, 2) there are $1\times10^8$ δTCS1$^+$ T cells (10% of total T cells in RA) per liter of blood in RA patients, 3) δTCS1$^+$ T cells sequestered in lymphoid tissues equals to that of blood, 4) it will require 15 μg of δTCS1$^+$ monoclonal antibody (Ab) for $10^8$ T cells to induce effective cell elimination or block of cell receptor function in vivo and 5) 5% of injected δTCS1$^+$ antibody is bioavailable, then the calculated dose is 2.1 mg per dose (see below.

$$\frac{(7000\text{ ml blood}}{\text{patient}} \times \frac{1\times10^8}{1000\text{ ml}} \times \frac{\text{cells}}{\text{blood}} \times \frac{15\ \mu\text{g Ab}}{10^8\text{ cells}} \times \frac{1}{.05} = \frac{2.1\text{ mg AB}}{\text{patient}}$$

10.2.5. REGIMEN

The in vivo half life of mouse $IgG_{2a}$ monoclonal antibody, known to one skilled in the art, averages around 4 hours. Therefore, a daily injection of about 2 mg per patient for 10 to 14 days is recommended in order to achieve optimal efficency.

10.3. SUMMARY

It is clear that lymphocytes are actively involved in the pathogenesis of Rheumatoid Arthritis (RA). Severe RA patients alone represent at least a million people in the United States. Although immunosuppression therapies, such as total lymphoid irradiation, and thoracic duct drainage, are efficacious for severe RA patients, but they are not suitable for all patients. To date, no satisfactory immunotherapy is available for severe RA patients who have failed or contraindicated for conventional second line drugs.

We have obtained evidence that about 1% of total T cells are involved in severe RA. An improved, novel, safer and efficacious immunotherapy of selectively eliminating the same 1% of total T cells in the body, using δTCS1 monoclonal antibody, has been developed. The best currently employed experimental drug abolishes 60% of T cells, leading to generalized immune suppression and raising serious issues relating to patient risk/benefit.

Studies of the infiltrated cells revealed that T cells represent the major cell type at the site of tissue injury, that is, the synovium of the RA joints. These T cells have been the main target for other immunosuppressive drugs. Several independent studies indicate that synovial T cells are primarily CD4+ helper T cells. Our data suggests that these synovial T cells bear two distinct types of T cell antigen receptors (TCR), namely, the αβ and the γδ TCR. Our evidence strongly suggests that a subset of γδ cells, identifiable by the δTCS-1 antibody, is significantly elevated in the blood and/or synovium of about 20–30% of RA patients. These patients tend to develop more severe forms of arthritis, i.e., Felty's Syndrome and RA with associated peripheral vasculitis. The lack of appropriate animal models, the historical excellent safety record of mouse monoclonal antibody products, and the severity of RA disease leads us to believe that it is timely to proceed with the drug development of the δTCS1 antibody as a useful therapeutic drug for arthritis patients.

It has been noted that δTCS1$^+$ T cells represent about 1% of total T cells in healthy individuals. They are postulated to regulate the development of αβ T cells via the lymphokines they secrete and to represent the motile population of γδ T cells in the body. The only FDA approved mouse monoclonal antibody, therapeutic OKT3, eliminates 100% of T cells, and the therapy requires supportive measures in a hospital setting for administration. However, with a 10–14 day course of daily OKT3 injections, the therapeutic effects lasted for about one to two years in treated patients who suffered from transplanted kidney rejection.

We propose to treat severe RA patients with δTCS1$^+$ monoclonal antibody by administering a daily I.V. dose of approximately 2 mg of the antibody for 0–14 days. The therapy is expected to be primarily maintained by outpatient administration.

## 11. EXAMPLE: MONOCLONAL ANTIBODIES REACTIVE WITH THE VARIABLE REGIONS OF α,β HUMAN T CELL ANTIGEN RECEPTOR ARE USEFUL IN THE TREATMENT OF RHEUMATOID ARTHRITIS

The first step needed in the development of T cell receptor specific therapeutics is to correlate specific T cell receptor gene usage with disease. Once it is known which T cell receptors (TCRs) are primarily involved in the disease, specific therapeutics can be produced.

A panel of TCR variable region genes were used to determine which variable regions correlate with rheumatoid arthritis. The data presented infra involves the analysis of rheumatoid arthritis patient samples using $V_\alpha$ and $V_\beta$ TCR gene probes. Similar analysis could also be done using $V_\gamma$ and $V_\delta$ genes as well.

11.1. MATERIALS AND METHODS

11.1.1. SAMPLES:

Paired synovial membrane derived T cell lines and peripheral blood T cell lines were prepared from 12 patients with RA (see Example 9, infra). Peripheral blood lines were also obtained from 5 normal individuals for controls using similar cell culture procedures.

11.1.2. T CELL RECEPTOR VARIABLE REGION GENE PROBES

There are 17 human $V_\alpha$ and 18 human $V_\beta$ subfamilies that have been identified to date (Toyonaga, B. and Mak, T. W., 1987, Annual Rev. Immunol., 5, 585–620). These Vβ subfamilies are named Vβ1 to Vβ 20. Subfamilies designated Vβ13 and Vβ14 have been merged into other families based upon the degree of sequence homology of the members. All except $V_{\alpha15}$ and $V_{\beta16}$ have currently been tested. In addition, there are about 5–10 human $V_\gamma$ (Forster, A., et al., 1987, EMBO, 6, 1945–1950) and 5–10 human $V_\delta$ (Takihara, Y., et al., 1989, J. Exp. Med., 169, 393) subfamilies that have been identified to date. As additional V α, β, γ, and δ regions become available, they may similarly be tested. Once correlations between disease and specific TCR V subfamilies

have been identified, the specific member of the subfamily responsible for the correlation can also be identified (see infra).

11.1.3. RNA PREPARATIONS:

RNA was isolated by the guanidinium isothiocyanate cesium chloride procedure (Maniatis, T., et al., 1982, In fMolecular Cloning: A Laboratory Manual", Cold Spring Harbor Laboratories, N.Y.). Total RNA was precipitated twice in 0.3 M sodium acetate and 2.5 volumes of ethanol. On average, 5 to 10 $\mu$g of total RNA was obtained from 10 million cultured T cells.

11.1.4. T CELL ANTIGEN RECEPTOR USAGE ANALYSIS:

The usage of T cell antigen receptor $\alpha$ and $\beta$ chains in the T cell lines was determined using 3 major steps; i) cDNA synthesis; ii) polymerase chain reaction amplification; and iii) DNA slot blot analysis.

11.1.4.1. cDNA SYNTHESIS:

Five $\mu$g of total RNA from each sample was primed for cDNA synthesis using the $C_\alpha$ olignucleotide and a $C_\beta$ oligonucleotide. To analyze TCR $\alpha,\delta$ gene usage, $C_\gamma$ and $C_\delta$ primers could be used in an analagous fashion. Both $C_\alpha$ and $C_\beta$ primers were 18-mers synthesized by New England Biolabs, Beverly, Mass. using the published sequences of the $\alpha$ and $\beta$ constant regions (Yanagi, Y., et al., 1984, Nature, 308, 145–149). The sequence of the $C_\alpha$ primer (5'-TTAGAGTCTCTCAGCTGG-3') is located 31 nucleotides 3' from the $NH_2$ terminus of the $\alpha$ chain constant region. The sequence flor the $C_\beta$ primer (5'-TTCTGATGGCTCAAACAC-3') is located 36 nucleotides 3' from the $NH_2$ terminus of the $\beta$ chain constant region. The $C_\beta$ oligonucleotide primed cDNA synthesis from both $\beta$ chain constant regions (Yanagi, Y., et al., 1984, Nature, 308, 145–149; Jones, N., et al., 1985, Science, 227, 311–314). The location of these primers was chosen such that the synthesized cDNA would comprise the variable, diversity, and joining regions of the T cell receptor mRNA and only a small portion of the constant region.

First strand DNA synthesis was performed according to published procedures (Okayama, H. and Berg, P., 1982, Mol. Cell. Biol., 2, 161–170; Gubler, U. and Hoffman, B. J., 1983, Gene, 25, 263–269) except that the reaction was terminated prior to synthesis of the second strand. The resulting templates were in the form of RNA:DNA hybrids. These duplexes were then used in an oligo-dG tailing reaction (Deng, G-R. and Wu, R., 1983, Meth, in Enzymol., 100, 96–117) which preferentially tails the 3' end of the cDNA strand over the RNA strand.

11.1.4.2. POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION:

The PCR reaction was performed in a thermocycler (Perkin-Elmer, Norwalk, Conn.) using recombinant Tag DNA polymerase (Cetus Corp., Emeryville, Calif.). Oligonucleotides, $d(C)_{10}$ and C$\alpha$ and C$\beta$, were used as primers for amplification. The PCR amplification procedure of Loh, E. Y., et al. (1989, Science, 243, 217–220) was used with the following modifications. PCR amplification was done for 30 cycles with each cycle comprising incubations at 92° C. for 1 minute, 50° C. for 1.5 minutes and 72° C. for 2.5 minutes. The last extension reaction was for 10 minutes at 72° C. All samples were amplified a total of 3 times with isolation of the amplified DNA fragment of about 300–400 base pairs between each round. The final amplified DNA samples were then precipitated with spermine to remove free nucleotides, before labeling with $^{32}P$ radiolabeled nucleotides. Labeling was done during 5 cycles of PCR amplification using all four $^{32}P$ labeled nucleotides at a ratio of 1:10 non-radiolabeled nucleotides. The resulting $^{32}P$ labeled DNAs were purified on elute-tip™ columns (Schleicher & Schuell, Keene, N.H.) to remove non-incorporated $^{32}P$ nucleotides.

11.1.4.3. DNA SLOT BLOT ANALYSIS:

DNA slot blots were prepared using a slot blot apparatus (Schleicher & Schuell, Keene, N.H.) and nylon membranes (Oncor, Gaithersburg, Md.) according to manufacturer's protocols. A panel of cDNA subclones comprising the variable region of $\alpha$ and $\beta$ chain TCR genes was spotted in duplicate on each slot blot (3 $\mu$g per slot). After the blots had been prepared containing the panel of TCR V region DNAS, individual blots were then hybridized to the $^{32}P$ labeled T cell derived cDNA generated in step #2. Individual patient samples were hybridized to duplicat blots. Hybridization condition and washes (Southern, E., 1979, J. Mol. Biol., 98, 503–517) were chosen to ensure no cross-hybridization between members of different subfamilies. The wash steps were performed at 42° C. in 0.2×SSC (30 mM sodium chloride, 3 mM sodium itrate, pH 7.4) with 0.1% sodium dodecyl sulfate using 4 washes of 20 minutes each. Following washing, the blots were blotted dry, and ther autoradiographed at −70° C. for 2–6 days using Eastman Kodak, X-Omat Xray film (Rochester, N.Y.). The developed autoradiographs were than scanned for intensity using a video densitometer (Model 620, Biorad Corp., Richamond, Calif.).

11.2. RESULTS

Even in a normal disease free state, the expression of TCRs varies for the different subfamilies. Some subfamilies, e.g. V$\beta$8, V$\beta$6 and V$\alpha$10, are expressed quite frequently and the expression of others is fairly rare. For disease correlation, the increased levels of expression in disease are determined relative to these base levels.

Using the cDNA synthesis, PCR amplification and slot blot hybridization procedure detailed in the materials and methods, paired RA samples including peripheral blood and synovial tissue derived T cell lines from each of 12 patients were analyzed relative to the expression in 5 normal peripheral blood controls. One basic assumption in this analysis is that the disease related T cells are most abundant at the site of the disease, e.g. the synovial membrane of patients with rheumatoid arthritis.

An example of this analysis is shown in FIGS. **18**. The left panel of FIG. **18**, shows the autoradiograph obtained when the T cell line ST-2 obtained from synovial tissue infiltrating lymphocytes was analyzed with the panel of V$\beta$ TCR genes. The right side of this figure shows the densitometry trace. In this cell line, it is clear that several TCR V$\beta$ genes (V$\beta$'s 2, 4, 6, 7, 8, 11 and 18) are expressed with V$\beta$4 being expressed in highest amounts. To determine which of these correlate with disease, this pattern of expression was compared to the pattern of expression observed in the peripheral ood derived T cell line (see FIGS. **19** and **20**).

FIG. **19** tabulates the results observed for V$\beta$ gene expression in each of the paired synovium tissue and peripheral blood derived T cell lines from the 12 RA patients analyzed. The X axis represents the number of patient samples (12 total) where a V$\beta$ was observed by the densitometry analysis as illustrated in FIG. **18** for the ST-2 cell line. The Y axis represents each of the 16 V$\beta$ gene probes tested. Peripheral blood data is represented by a crosshatched bar and synovial tissue data is represented by an open bar for each V$\beta$. From this figure, it can be determined that in the 12 RA patient samples analyzed, V$\beta$3, V$\beta$9, V$\beta$10 and V$\beta$12 were expressed more often in the synovial tissue derived T cell lines than in the peripheral blood derived T cell lines. For example, the ratio of presence in synovium to presence in the peripheral blood sample was found to be 1.4 V$\beta$3. By this analysis, the most frequently expressed V$\beta$ genes in the synovium relative to the peripheral blood were V$\beta$3, V$\beta$9, V$\beta$10 and V$\beta$12.

When the same data was analyzed as shown in FIG. **20**, the frequently used genes were V$\beta$1 (ratio=4.0), V$\beta$3 (ratio=infinity), V$\beta$6 (ratio=3.0), V$\beta$9 (ratio=infinity), and V$\beta$10 (ration=infinity). For the analysis in FIG. **20** only the domi-

nant Vβ in each sample as determined by the desitometry trace was used; the assumption being that although the T cell line may contain varying subpopulations of T cells, the dominant subpopulation could be the most relevant one. The frequencies of Vβ3, Vβ9, and Vβ10 were high when the data from the 12 patients was analyzed either for total expression or dominant expression.

When the same samples were analyzed for total Vα gene expression (FIG. **21**), the results were less clear. The reason for this turned out to be that 85% of the synovium or peripheral blood derived T cell lines analyzed preferentially used Vα10 (FIG. **22**). Although other Vα's were also represented in the cell line populations. Vα10 was by far the dominant one with the densitometry peak height for Vα10 being 100 fold greater than those of the other 15 Vα genes. This raises the possibility that Vα10 may represent a universal Vα that can pair well with most Vβ chains. FIG. **21** shows that Vα12 (ratio=infinity) may be the next most commonly expressed Vα gene in synovium, but its level of expression is low when compared to the level of expression of Vα10.

11.3. SUMMARY

This analysis has shown that T cell populations at the site of disease, e.g. the joint synovial membrane, appear to predominantly express specific Vβ chains. One mechanism of autoimmunity may be that disease-related autoantigens are recognized by the body's own T cells via specific T cell antigen receptor α, β, γ and δ chains. After antigen recognition, these T cells clonally expand to give rise to an oligoclonal population of disease-related T cells. Other mechansisms that may be involved include recruitment of specific cells to the disease site which would then represent an oligoclonal population of cells. In the total population of cells present at the disease site, the oligoclonal cells can be detected, as they will be using the TCR variable regions that are most frequently expressed. To date, our study has shown that the most frequently expressed Vβ genes in the synovial membrane of 12 RA patients were Vβ3, Vβ9, and Vβ10 and Vδ1 was preferentially used in the γδ+ T cells present in synovial fluid. To refine this correlation even more, patient HLA type, disease state and expression of TCR genes for α, β, γ and δ chains and for TCR Diversity-Joining region expression may be determined. It is expected that as patients are subgrouped by HLA type, the disease correlations will become even stronger.

11.4. DISCUSSION: TREATMENT OF RHEUMATOID ARTHRITIS

PATIENTS WITH TCR α, β SPECIFIC REAGENTS

Once a disease correlation has been made between a disease state and specific TCR gene expression, then the next step is to develop the TCR specific therapeutics. One class of such therapeutics are anit-TCR antibodies.

For the analysis presented supra on the preferential use of 3 Vβ genes in rheumatoid arthritis patients, it is envisioned that a specific therapeutic would involve a multiple antibody cocktail of anti-TCR antibodies specific for Vβ3, Vβ9 and Vβ10. This therapeutic would thus target only the T cell subsets expressing these 3 Vβ TCRs and not effect other non-expressing T cells.

## 12. DEPOSIT OF HYBRIDOMAS

The following hybridoma cell lines, producing the indicated monoclonal antibody, have been deposited with the American Type Culture Collection, 10801 University Blvd., Manassas, Va. 20110-2209 and have been assigned the listed accession numbers:

| Hybridoma | Monoclonal Antibody | Accession Number |
|---|---|---|
| δTCAR-3 | δTCS1 (δTCAR-3) | HB 9578 |
| 3A8 | αF1 | HB 9900 |
| 3D6 | αF2 | HB 9901 |
| W112 6G-2 | W112 | HB 9927 |
| 2D1 | 2D1 | HB 9928 |
| 5.A6.E9 | Anti-TCRδ1 | HB 9772 |
| RS2A-2-H-7 | δTCS1, isotype IgG2a | HB 10110 |

The present invention is not to be limited in scope by the cell lines deposited since the deposited embodiments are intended as single illustrations of one aspect of the invention and any cell lines which are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

What is claimed is:

**1**. A method of detecting a Vβ5.3 T cell, comprising:

(a) contacting a tissue specimen with a monoclonal antibody that binds to an epitope of the Vβ5.3 variable region of the beta chain of a T cell antigen receptor, wherein said monoclonal antibody is W112; and

(b) detecting whether immunospecific binding has occurred.

**2**. The method of claim **1** wherein the monoclonal antibody is detectably labeled.

**3**. The method of claim **1** wherein the monoclonal antibody is bound to a therapeutically useful molecule.

**4**. The method of claim **1**, **2** or **3** in which the tissue is contacted in vitro.

**5**. The method of claim **1**, **2** or **3** in which the tissue is contacted in a histologic specimen.

**6**. The method of claim **1**, **2** or **3** in which the tissue is contacted in vivo.

**7**. A method for diagnosing rheumatoid arthritis, comprising:

(a) contacting a sample obtained from a patient with a monoclonal antibody that binds to an epitope of the variable region of the beta chain of a T cell antigen receptor; and

(b) detecting whether immunospecific binding has occurred, detection being indicative of rheumatoid arthritis.

**8**. The method of claim **7** wherein the monoclonal antibody is detectably labeled.

**9**. The method of claim **7** wherein the monoclonal antibody is bound to a therapeutically useful molecule.

**10**. The method according to claim **7**, **8** or **9** in which the sample is selected from the group consisting of peripheral blood, synovial tissue, and synovial fluid.

**11**. The method according to claim **7**, **8** or **9** which is performed in vitro.

**12**. The method according to claim **7**, **8** or **9** which is performed in vivo.

* * * * *